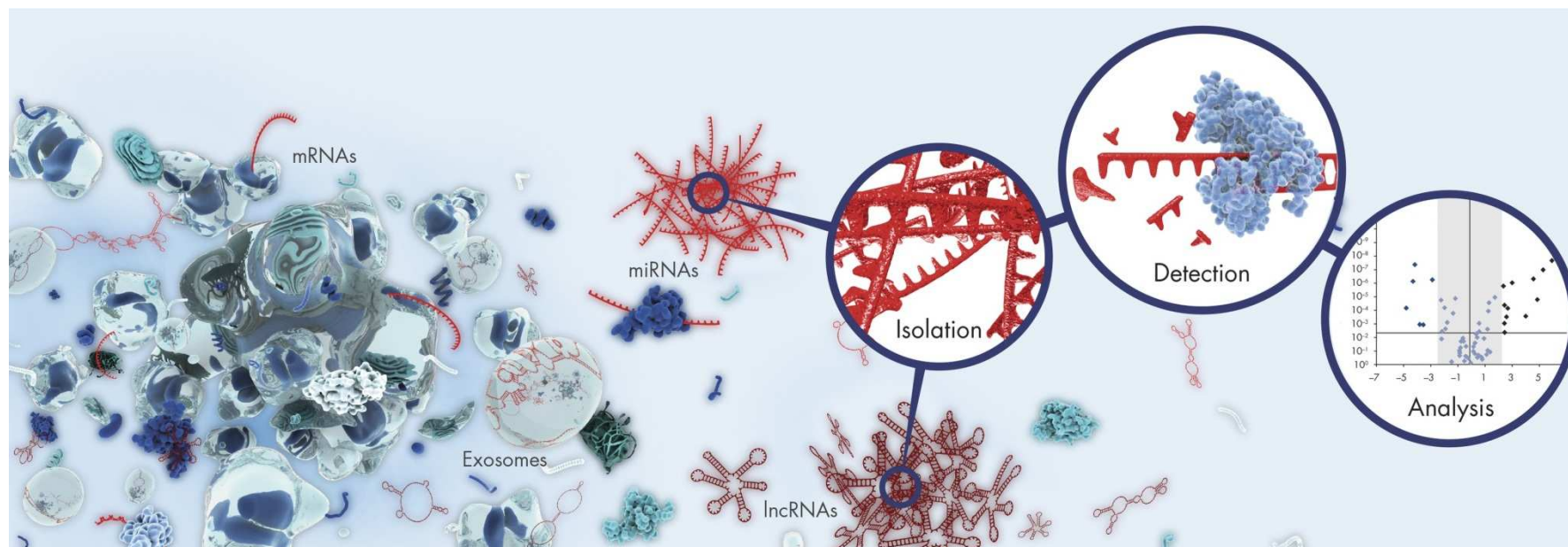


Introduction To Real-Time Quantitative PCR (qPCR)



Webinar-related questions:
QIAwebinars@QIAGEN.com

Technical Support:
BRCsupport@qiagen.com



Welcome to our 4-part webinar series on qPCR

qPCR technology overview, applications, data analysis and interpretation

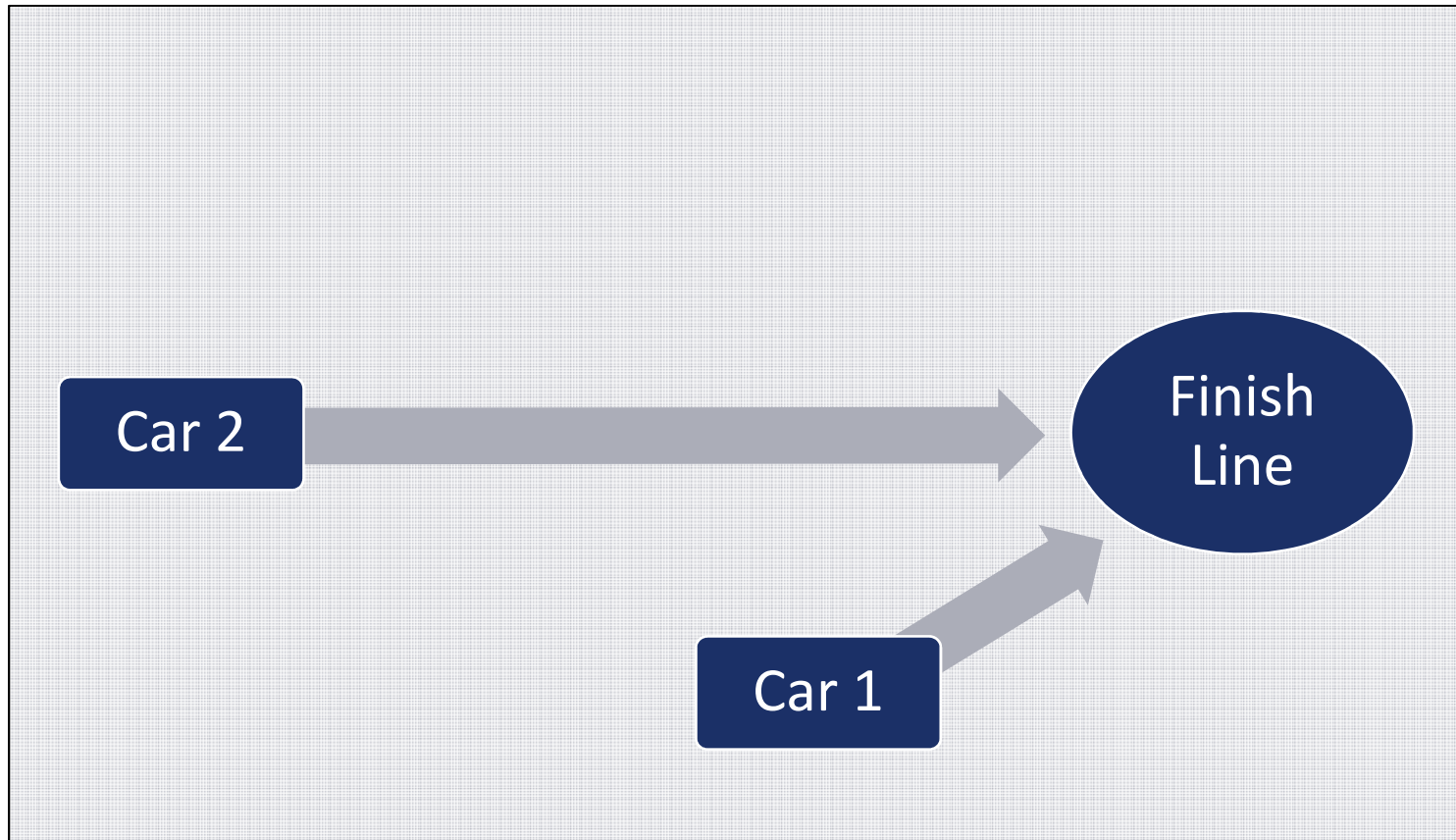


Registration link: <https://attendee.gotowebinar.com/register/1255411748535841794>

- ☐ Part 1: Introduction to Real Time PCR (Q-PCR/qPCR/qrt-PCR)
- ☐ Part 2: Pathway-focused Gene Expression Analysis - Advanced Real-Time PCR Array Technology
- ☐ Part 3: PCR Array Data Analysis Tutorial
- ☐ Part 4: Accelerate your discovery with QIAGEN service solutions for biomarker research



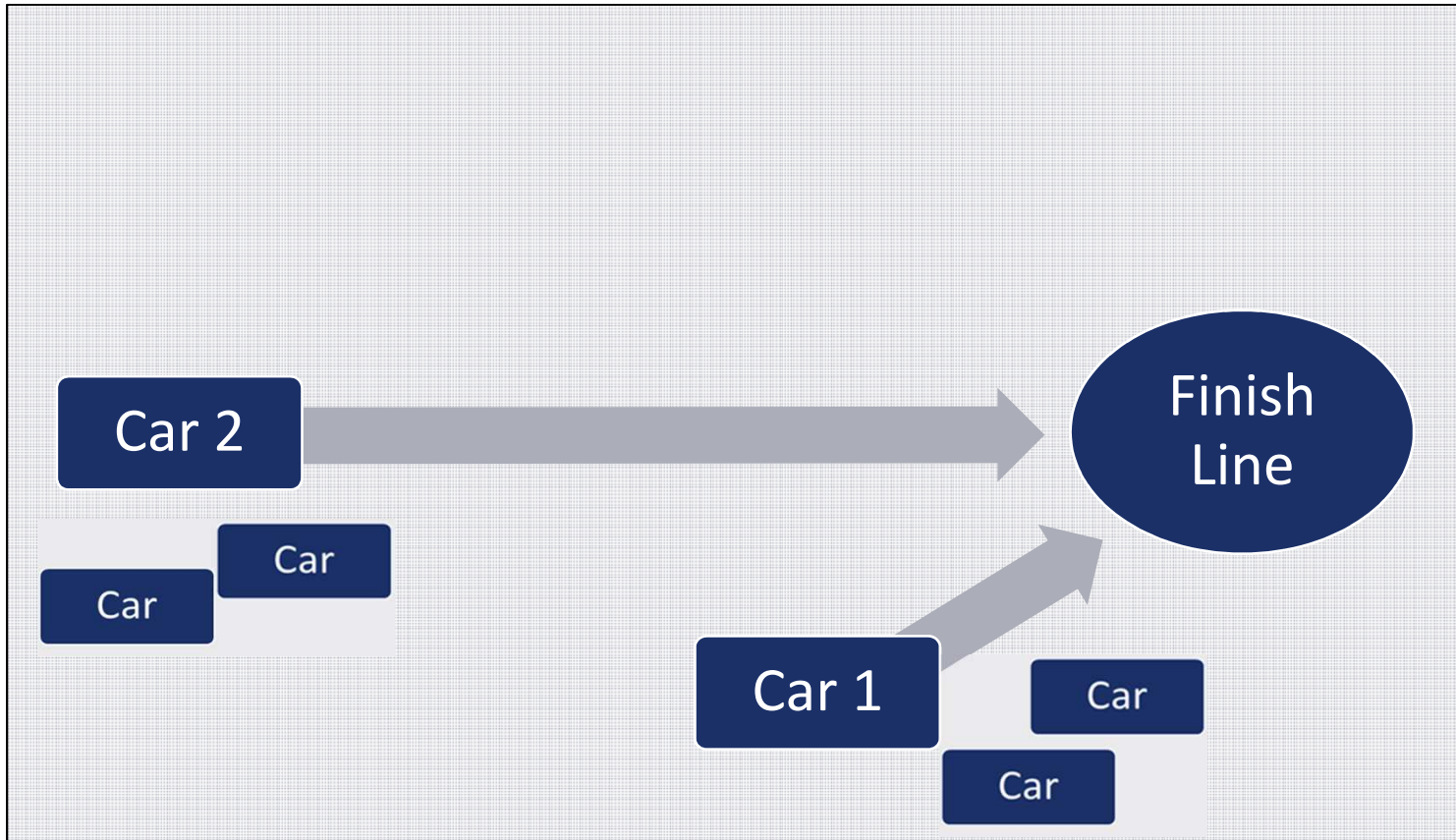
- QIAGEN products shown here are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.
- For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.QIAGEN.com or can be requested from QIAGEN Technical Services or your local distributor.



Question: How far apart are the 2 cars?

- Cars race at same speed to finish line
- As car 1 crosses finish line, calculate time for car 2 to finish
- Calculate difference in starting position mathematically ($d = \text{rate} \times \text{time}$)

How does qPCR work?



Question: How far apart are the 2 cars?

- Many cars; how to differentiate cars of interest



1. What is qPCR? Applications and workflow
2. qPCR for gene expression: What is the change in gene expression during differentiation?
3. Factors Critical For A Successful qPCR Assay
4. RNA purity and integrity
5. Reverse Transcription
6. qPCR in Action
7. Reporter chemistries
8. Characteristics of a good qPCR assay
9. Analyzing qPCR curves
10. Data & analysis



What is qPCR? Applications and workflow

What does Real-Time qPCR stand for?

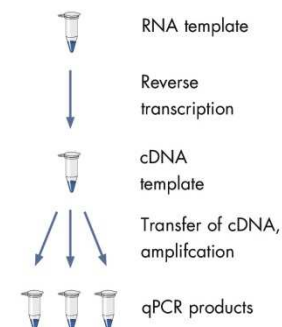
- Quantitative Polymerase Chain Reaction (qPCR) is a **sensitive and reliable** method for detection and quantification of nucleic acid (DNA & RNA) levels.
- It is based on **detection and quantification of fluorescence** emitted from a reporter molecule **in real time**.
- This detection occurs during the accumulation of the PCR product **with each cycle of amplification**
- Monitor the **PCR reaction during early & exponential phase**, where the first significant increase in the amount of PCR product correlates to the initial amount of target template.

Applications for qPCR

RNA

- Gene Expression Profiling Analysis
- miRNA Expression Profiling Analysis

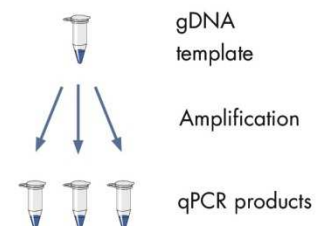
cDNA analysis —
two-step qRT-PCR



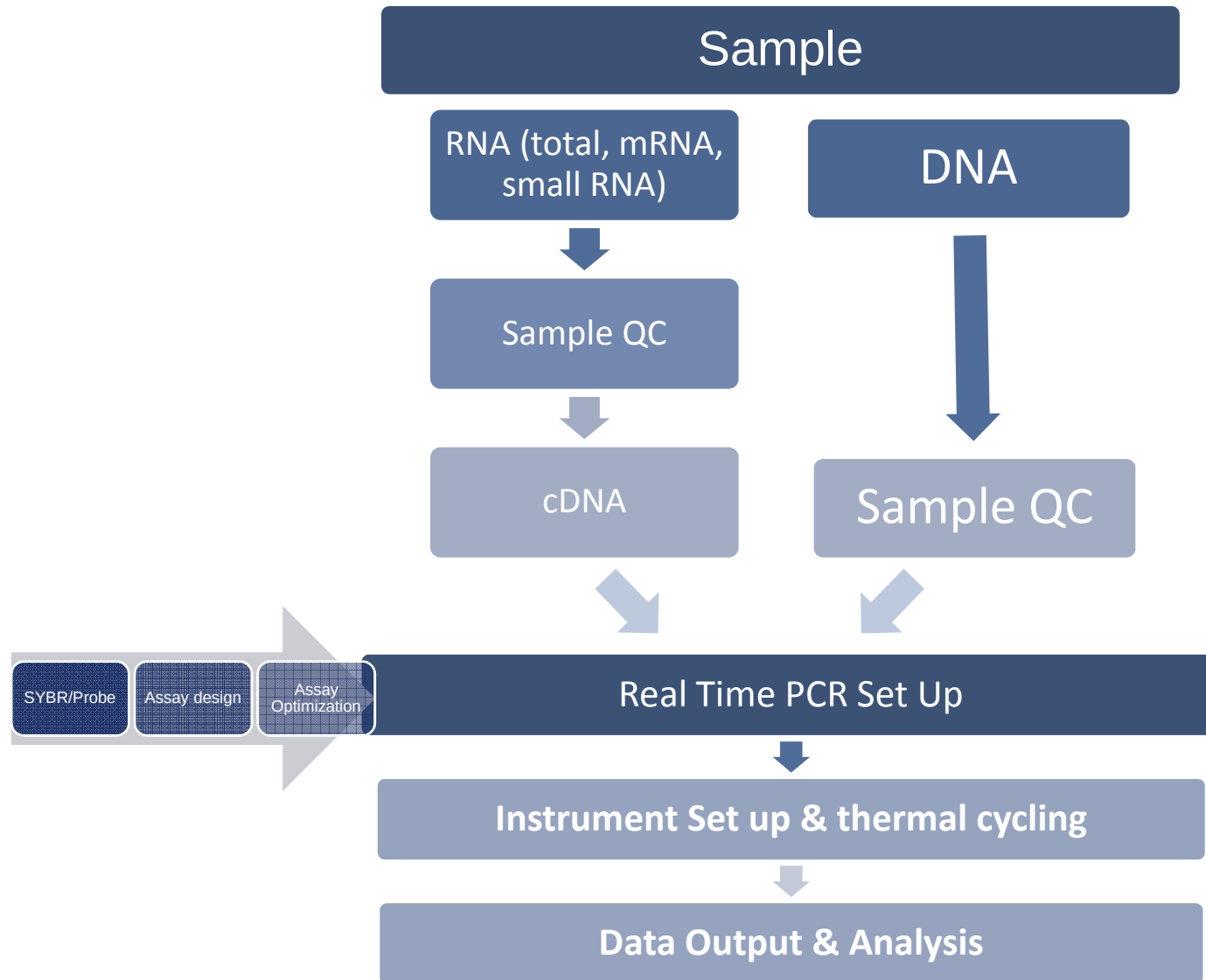
DNA

- SNP Genotyping & allelic discrimination
- Somatic Mutation Analysis
- Copy Number Detection/Variation Analysis
- Chromatin IP Quantification
- DNA Methylation Detection
- Pathogen Detection
- Viral Quantification

gDNA analysis —
qPCR



Work Flow: A Brief Look



Applications for qPCR

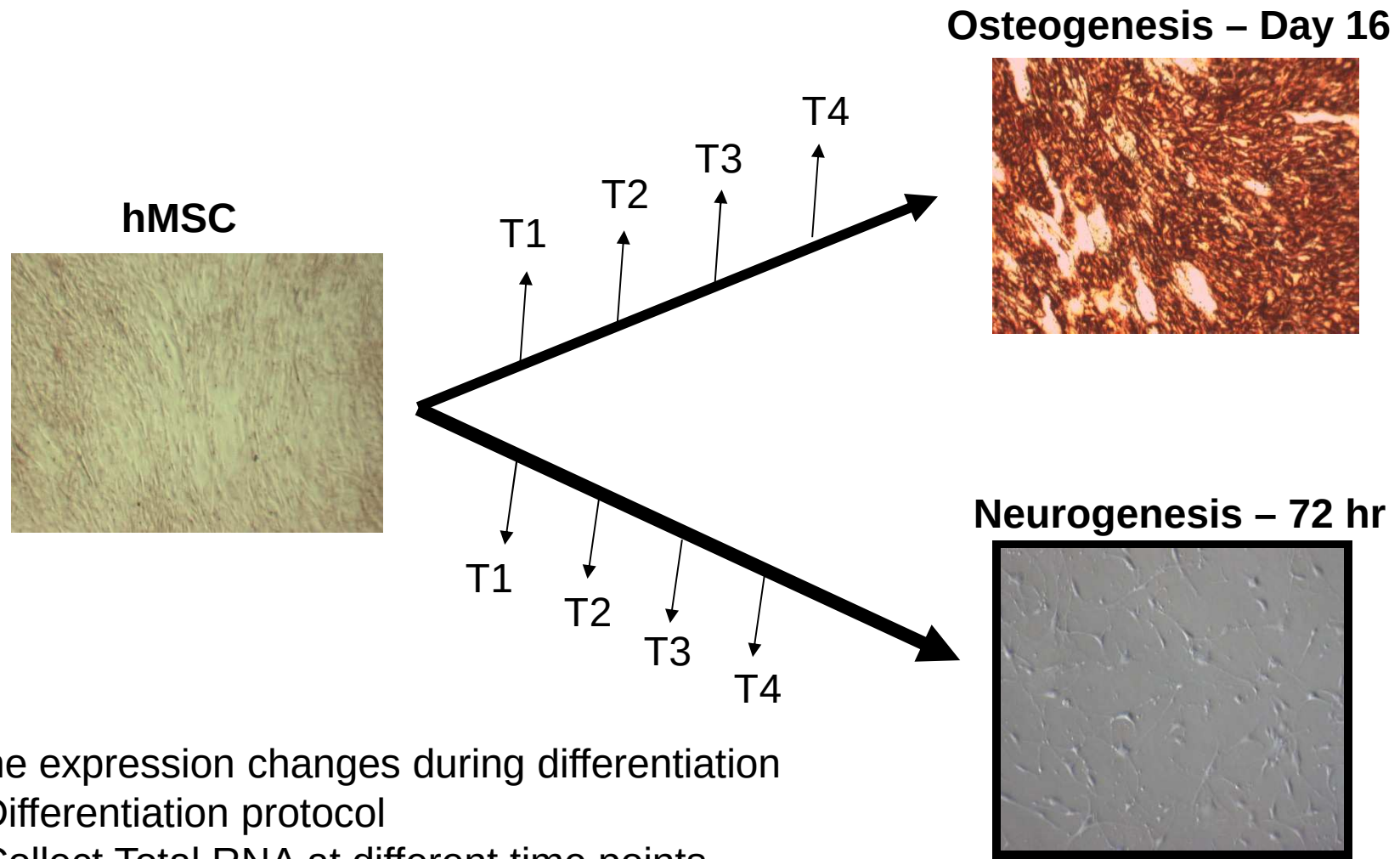
RNA

- [Gene Expression Profiling Analysis](#)
- miRNA Expression Profiling Analysis

DNA

- SNP Genotyping & allelic discrimination
- Somatic Mutation Analysis
- Copy Number Detection/Variation Analysis
- Chromatin IP Quantification
- DNA Methylation Detection
- Pathogen Detection
- Viral Quantification

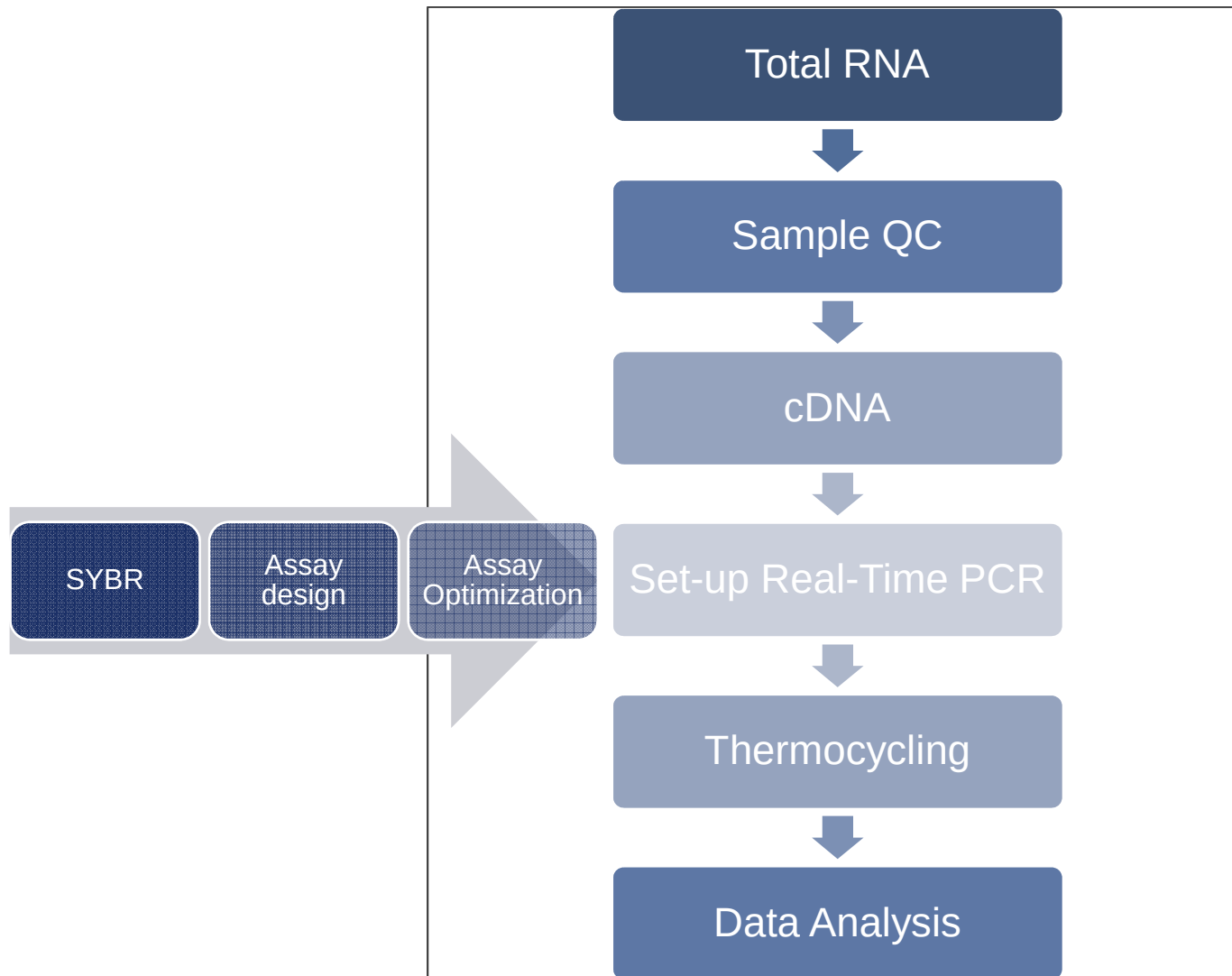




Gene expression changes during differentiation

- Differentiation protocol
- Collect Total RNA at different time points
- Measure 1 HKG and 1 GOI (TNF α)
- Repeat experiment 3x (biological replicates)

Work Flow: Gene expression profiling



Factors Critical For A Successful qPCR Assay

- A. DNA or RNA sample preparation - Template quality
 - Appropriate sample prep kits/reagents
 - Inhibitors can compromise RT or PCR
- B. Reverse transcription to convert RNA to cDNA
 - Choose RT kits
 - type of RT
 - which type of primers
 - controls?
- C. Assay design: chemistry, specificity, PCR efficiency, & throughput & cost
 - Choose validated assay, or need to validate our own?
- D. Running PCR
 - Commercial mastermix or make own (primer, probe, master mix)
- E. Data analysis tool
 - User friendly
 - Streamlined data analysis module



- RNA Isolation:
 - Considerations
 - Sample type/amount
 - Target (total RNA, mRNA, miRNA)
 - Throughput
 - Difficult samples (stool, FFPE)
 - Challenges
 - Contamination (gDNA)
 - Yield
 - Quality
 - Method
 - Qiazol?
 - Column based method (RNeasy?)
 - Both: Efficient lysis and inhibition of RNases; molecular grade RNA
 - miRNA? Use a kit specific for miRNA and mRNA

miRNeasy mini Kit



Qiazol:
phenol/guanidine-based lysis

Instant inactivation of RNases
Instant end of biological activities

Column cleanup:
Molecular biology grade RNA

Archive miRNA for next project

Sample Quality

Purity/ Quantity:

Spectrophotometer: measure 260/280 and 260/230

- OD260 is used to calculate amount of nucleic acid
 - 260/280 ratio
 - typical minimum value 1.8-2.0
 - 260/230 ratio
 - typical minimum value 1.7
- Low ratio may indicate a contaminant: protein, QIAzol, Carbohydrates, Glycogen
- Absorbance measurements do not show integrity of RNA

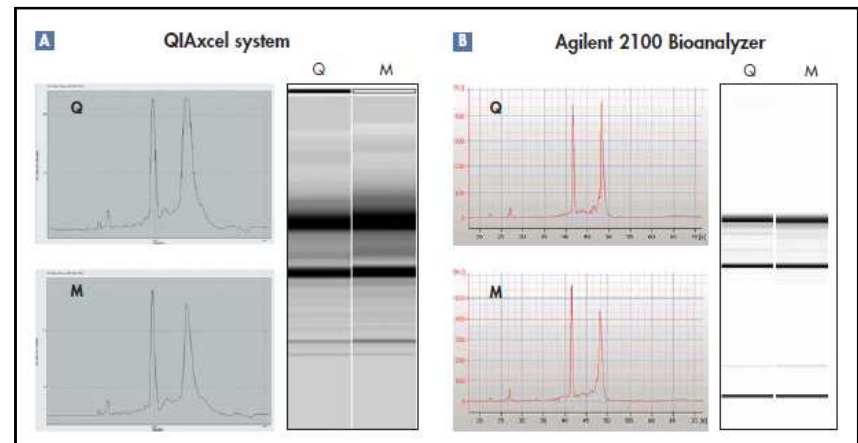
Integrity:

Denaturing RNA Agarose Gel

- Usually through ribosomal bands

QIAxcel/ Bioanalyzer

- Capillary electrophoresis
- Automate RNA integrity analysis
- RNA integrity analysis number



Factors Critical For A Successful qPCR Assay

qPCR Components

A. Templates:

- RNA
 - Starting amount ~10-1000 copies of NA per qPCR assay
 - For a low-expressed gene, need 10ng equivalent of RNA per reaction
 - Want to start with about 100pg to 1ug RNA
- Reverse Transcription
 - One-Step or Two-Step Reaction

B. Primers/Probes

C. Master Mix

- DNA polymerase
- Mg⁺⁺
- dNTPs
- Buffer
- Passive reference dye

D. Cycling Conditions

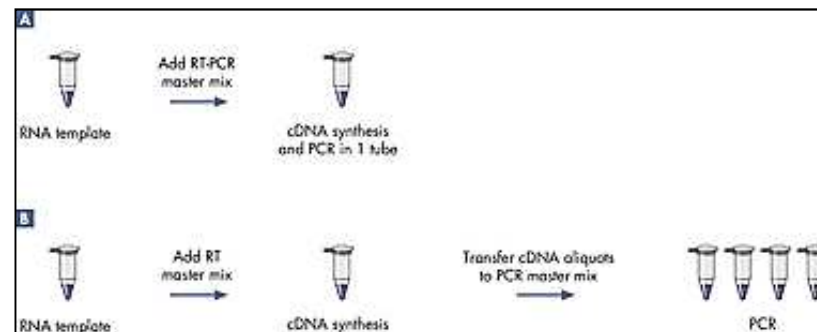
- Denature>Annealing>Extension
- Denature>Annealing/Extension

One-Step PCR

- 1 Tube Reaction

Two-Step PCR

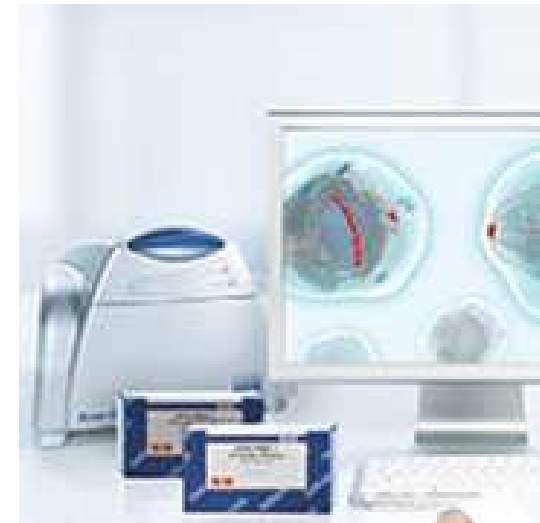
- 2 separate reactions
- RT Reaction
- qPCR Reaction



Reverse Transcription: Used to make cDNA copy of RNA

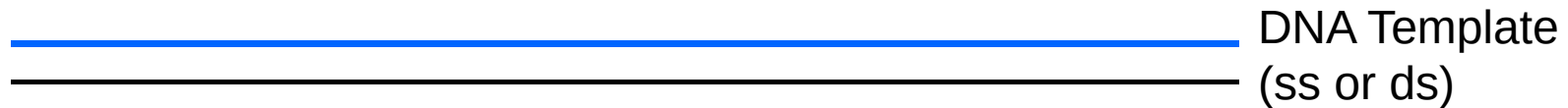
Reagents:

- Reverse transcriptase – many different kinds
- dNTPs
- Buffers for RT
- Primers
 - Random pentamers or hexamers
 - Oligo-dT
 - Both
- Control RNA to monitor reverse transcription kit?



Important Notes:

- RT reaction is linear
- Do not try to reverse transcribe too much RNA
- Sensitivity of qPCR step is dependent on good RT reaction
- Monitor RT reaction for equal efficiency across samples

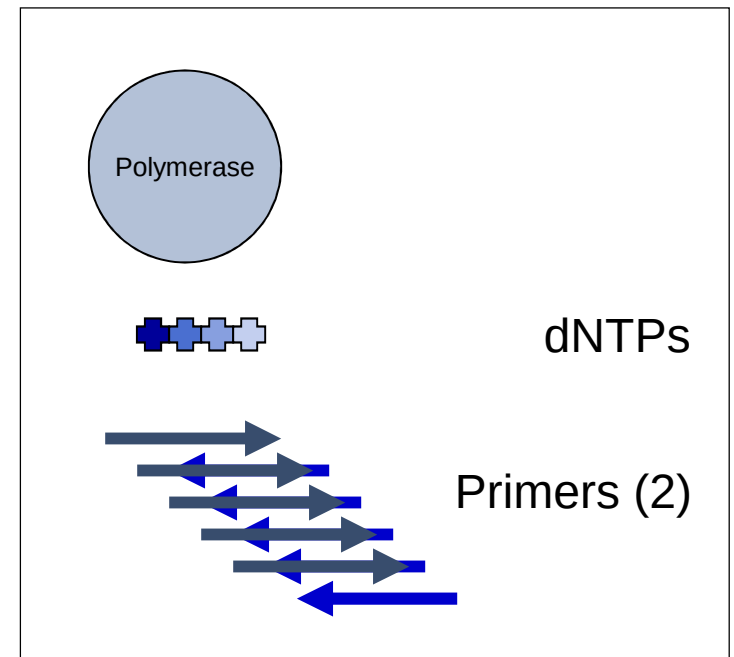


What is in a PCR Reaction?

PCR= Polymerase Chain Reaction
Exponential Amplification of DNA in single tube
All reagents in excess (non-limiting)

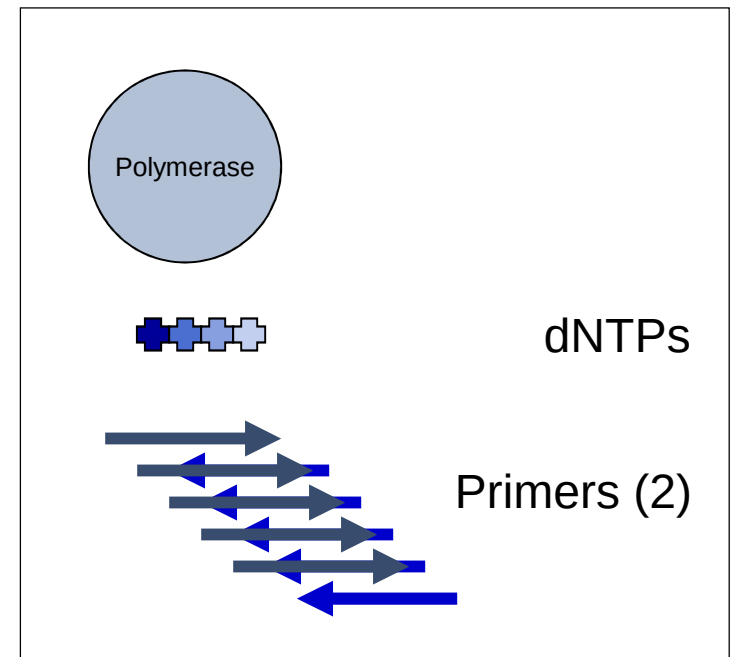
Components:

- Thermostable polymerase
- dNTPs
- Primers
- Template



————— DNA Template
————— (ss or ds)

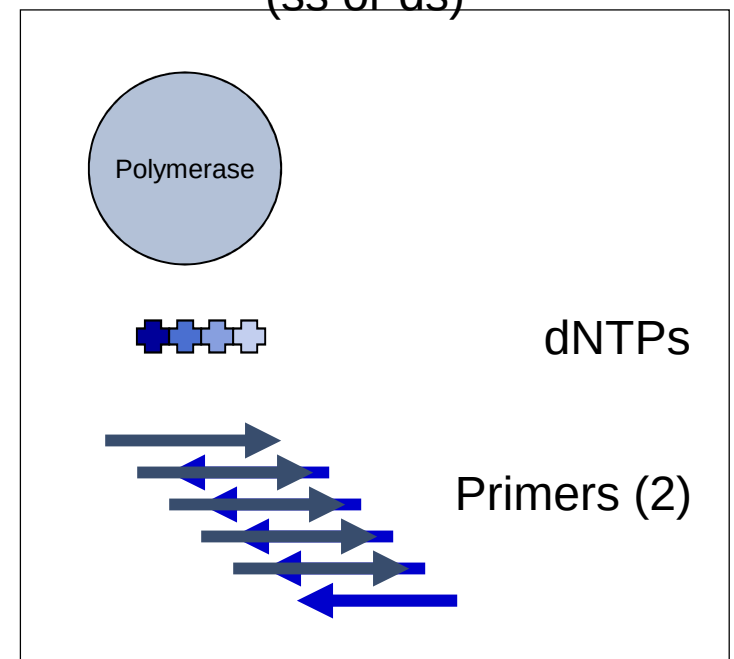
1. Heat denature template (~95C)
2. Annealing (~60C)
3. Extension (~60C)
4. Repeat (~95C)

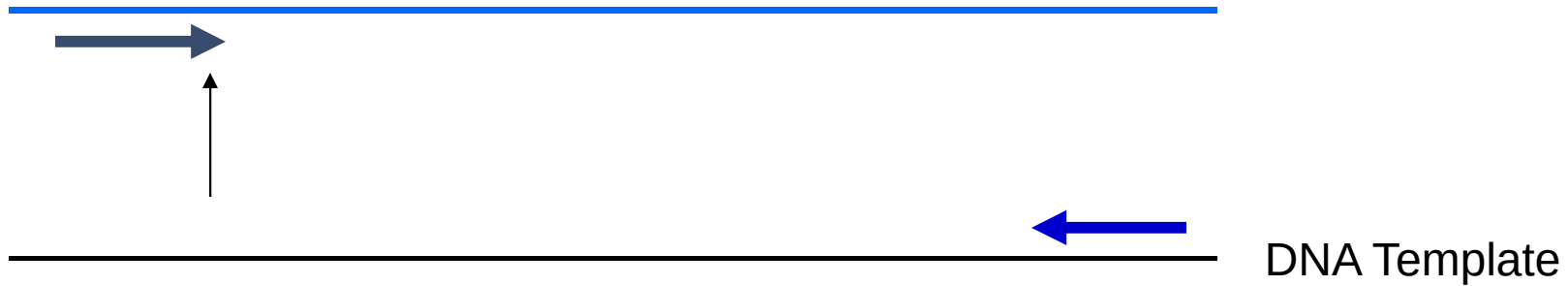


↑
Heat denature

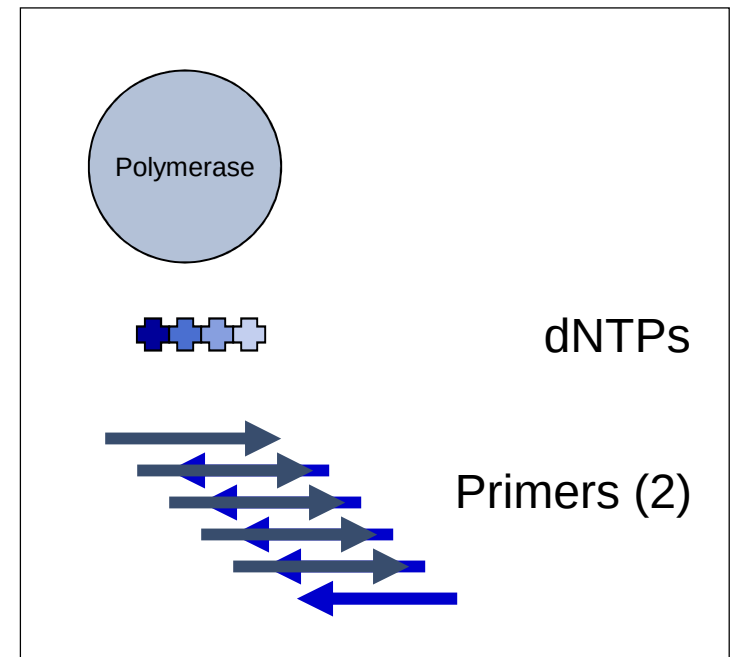
1. Heat denature template (~95C)
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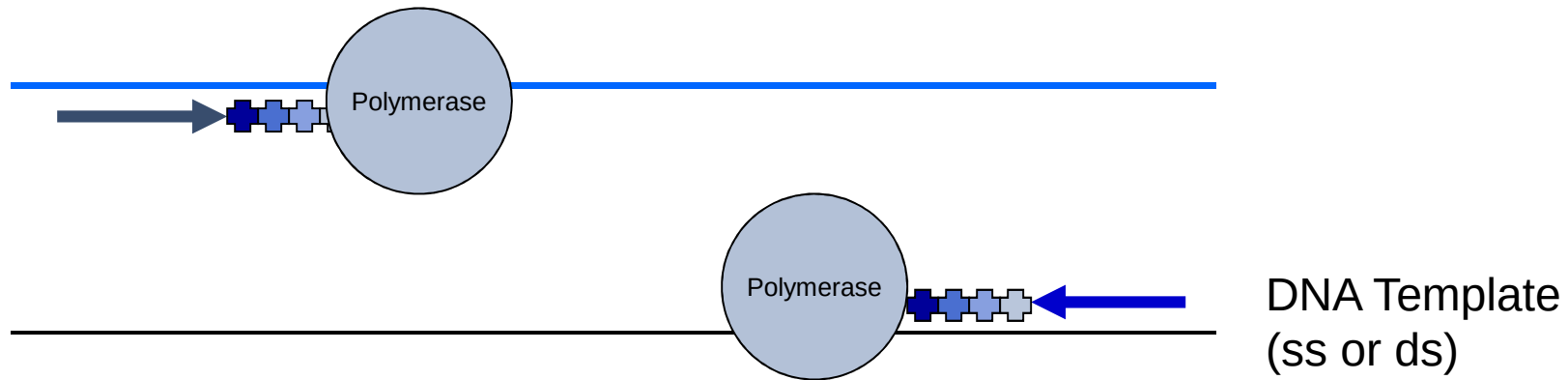
DNA Template
(ss or ds)



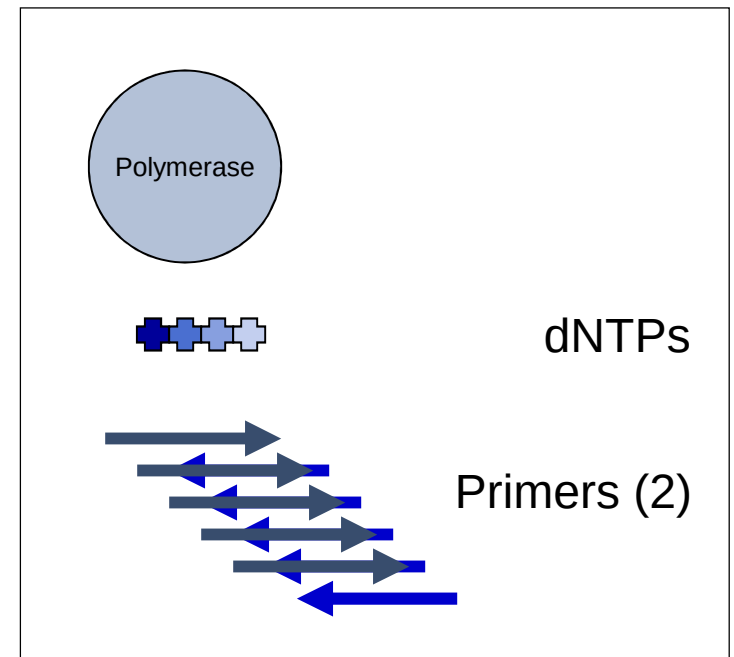


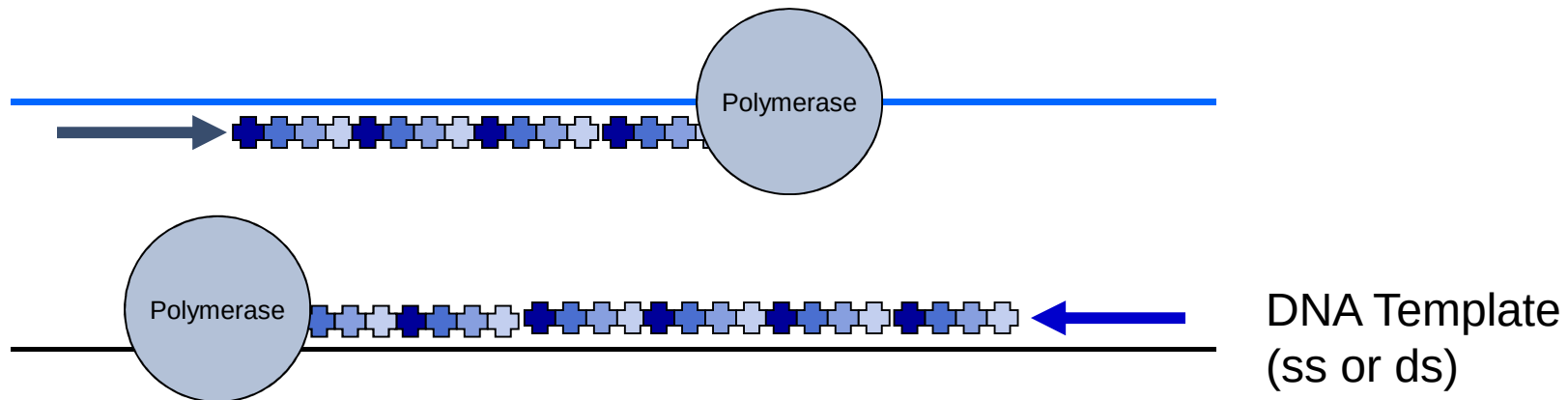
1. Heat denature template (~95C)
2. **Annealing (~60C)**
3. Extension (~60C)
4. Repeat (~95C)



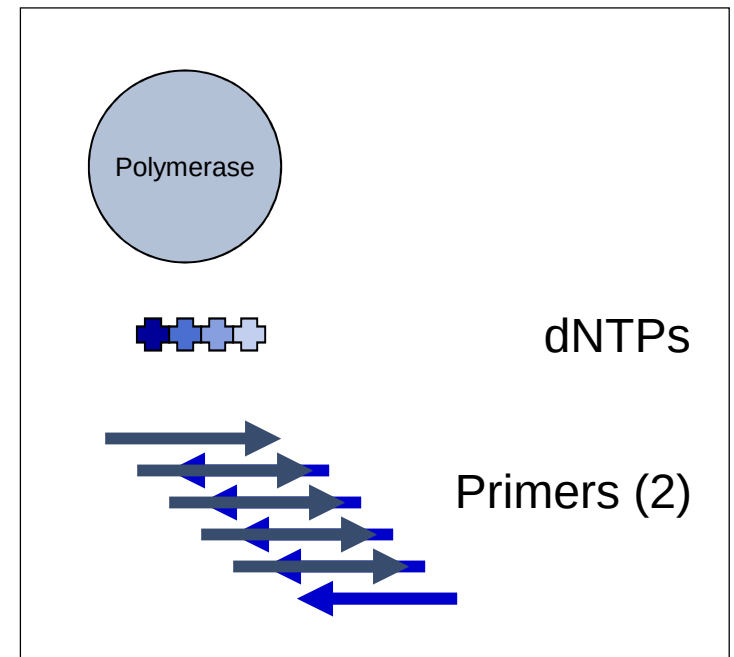


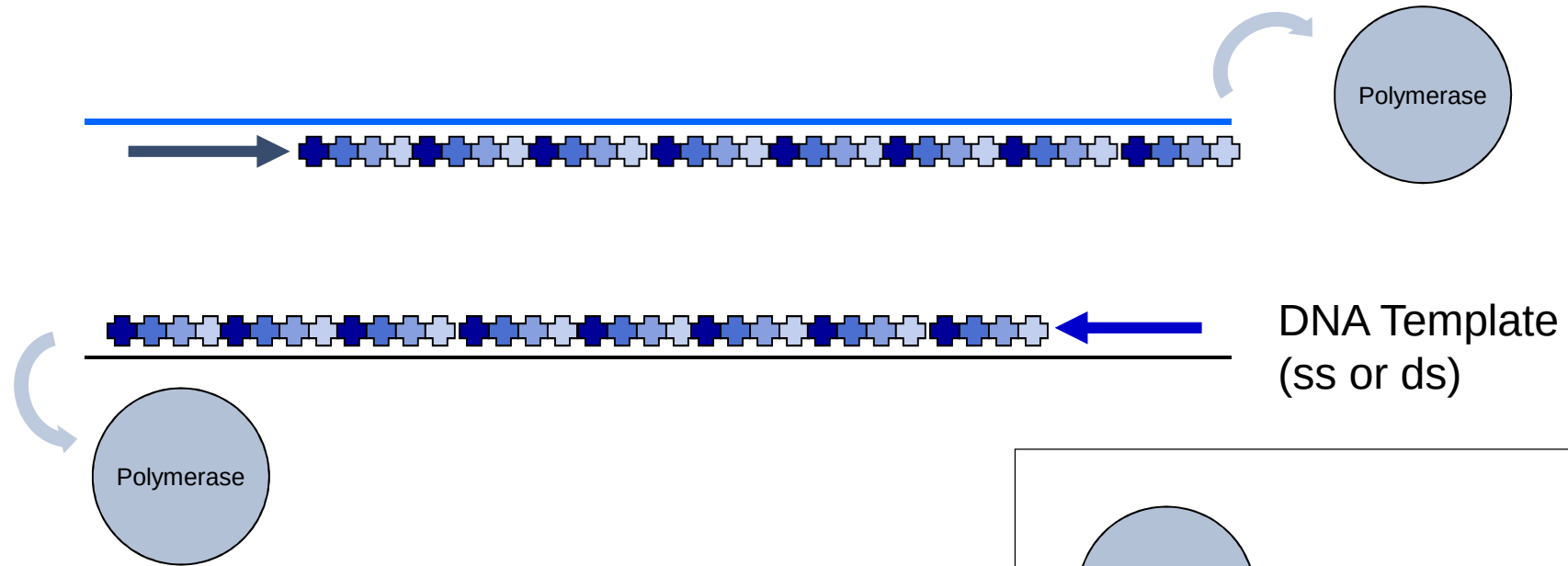
1. Heat denature template (~95C)
2. **Annealing (~60C)**
3. Extension (~60C)
4. Repeat (~95C)



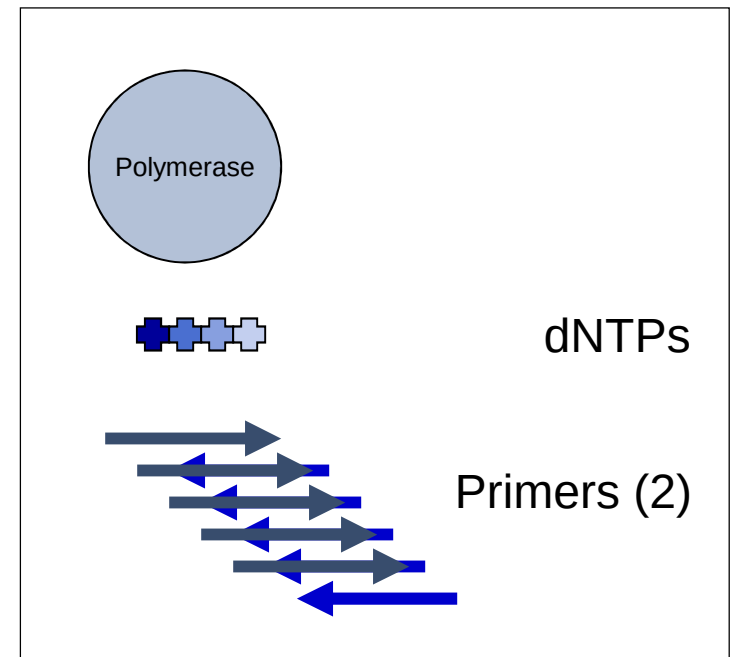


1. Heat denature template (~95C)
2. Annealing (~60C)
3. Extension (~60C)
4. Repeat (~95C)



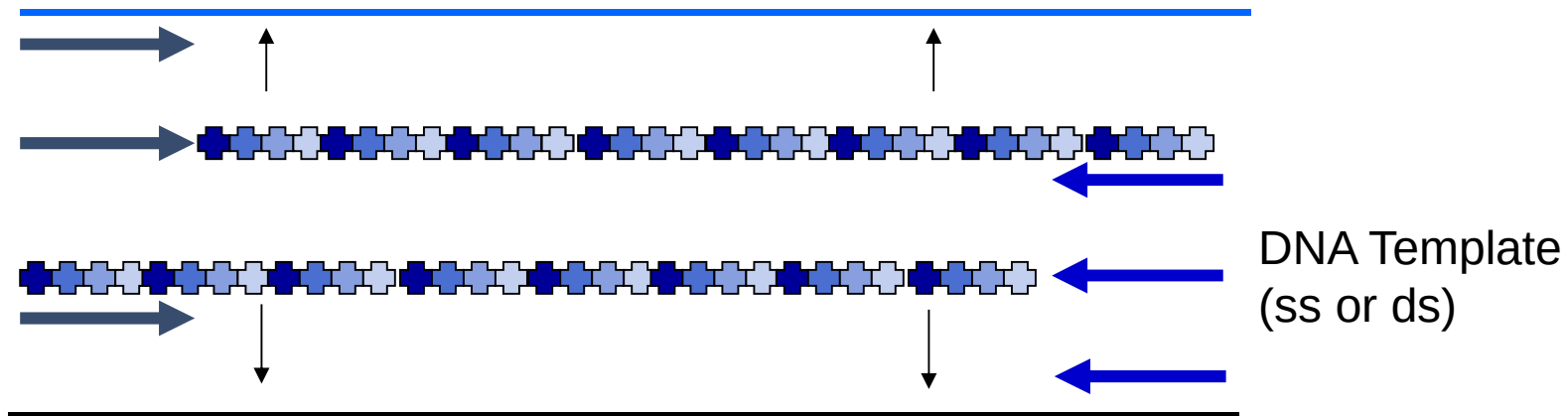


1. Heat denature template (~95C)
2. Annealing (~60C)
3. Extension (~60C)
4. Repeat (~95C)

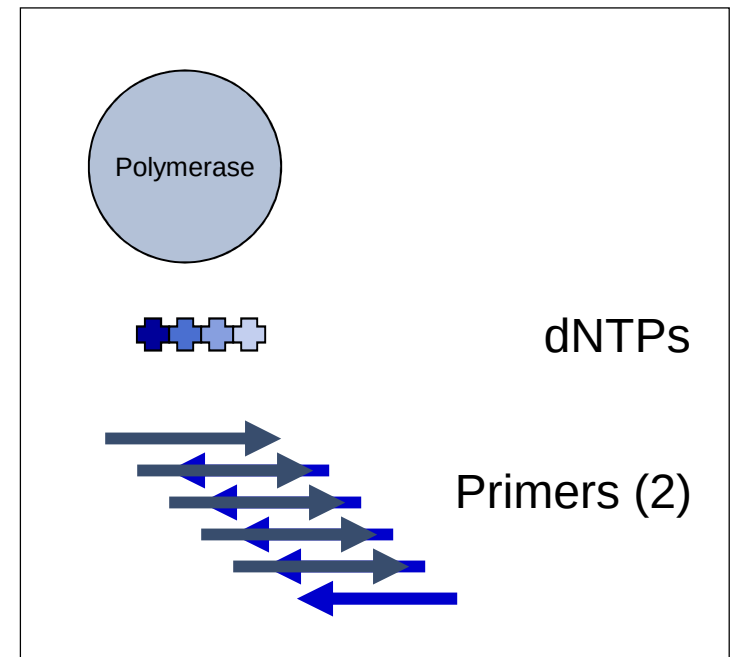


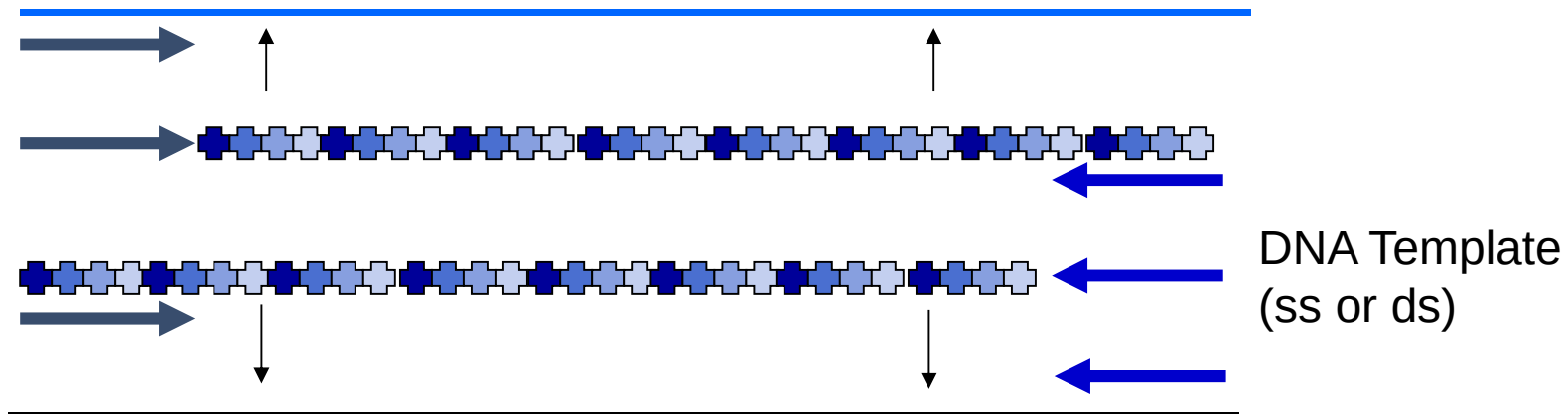


qPCR in Action



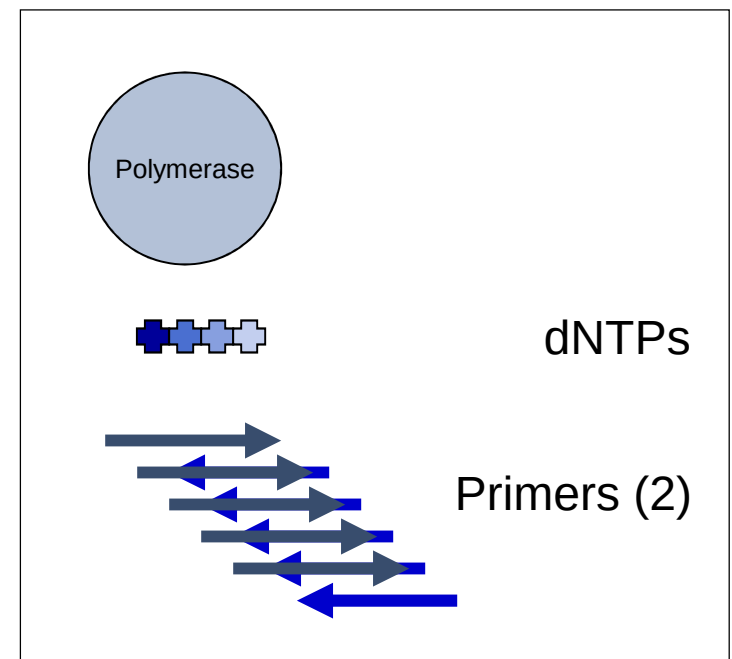
1. Heat denature template (~95C)
2. Annealing (~60C)
3. Extension (~60C)
4. Repeat (~95C)





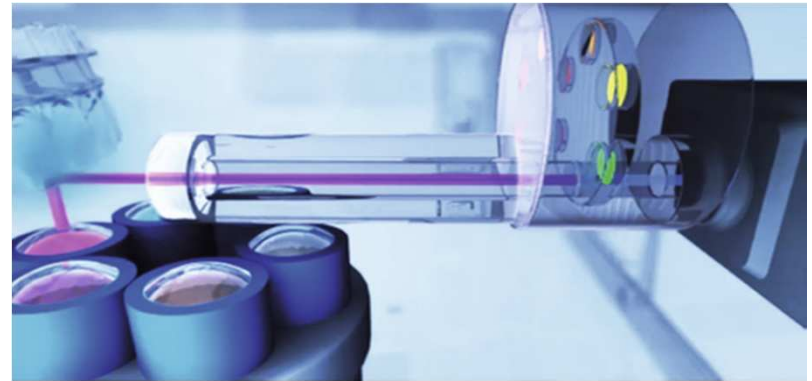
How do you make this a quantitative PCR?

- Measure DNA amount at end of each cycle to get ratio of DNA or absolute amount (if using a standard)
1. Heat denature template (~95C)
 2. Annealing (~60C)
 3. Extension (~60C)
 4. Measure amount of PCR Product
 5. Repeat (~95C)

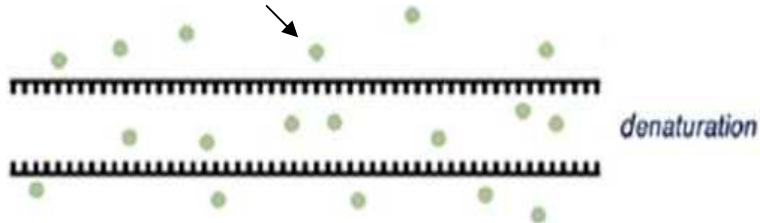


Real-Time qPCR Fluorescence Chemistry

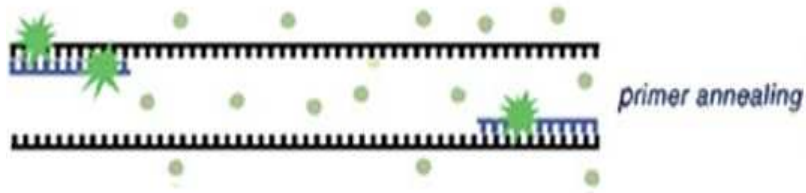
- DNA binding agents
 - SYBR® I Dye
- Hydrolysis Probes
 - Dual-labeled Hydrolysis (Taqman®) probe
- Others, such as hybridization probes
 - Molecular beacon and scorpion probes



Non fluorescent SYBR I

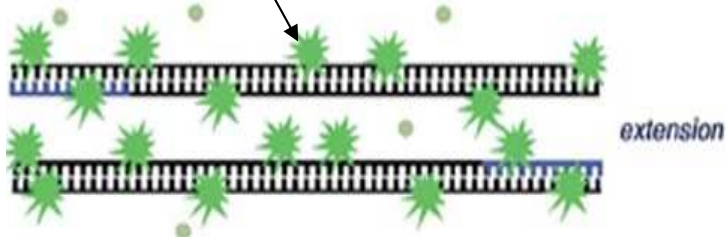


SYBR I binds to double-strand DNA but not single strand DNA. Little fluorescence emitted from SYBR I in solution.



SYBR I upon binding to double-strand DNA emits fluorescence very brightly

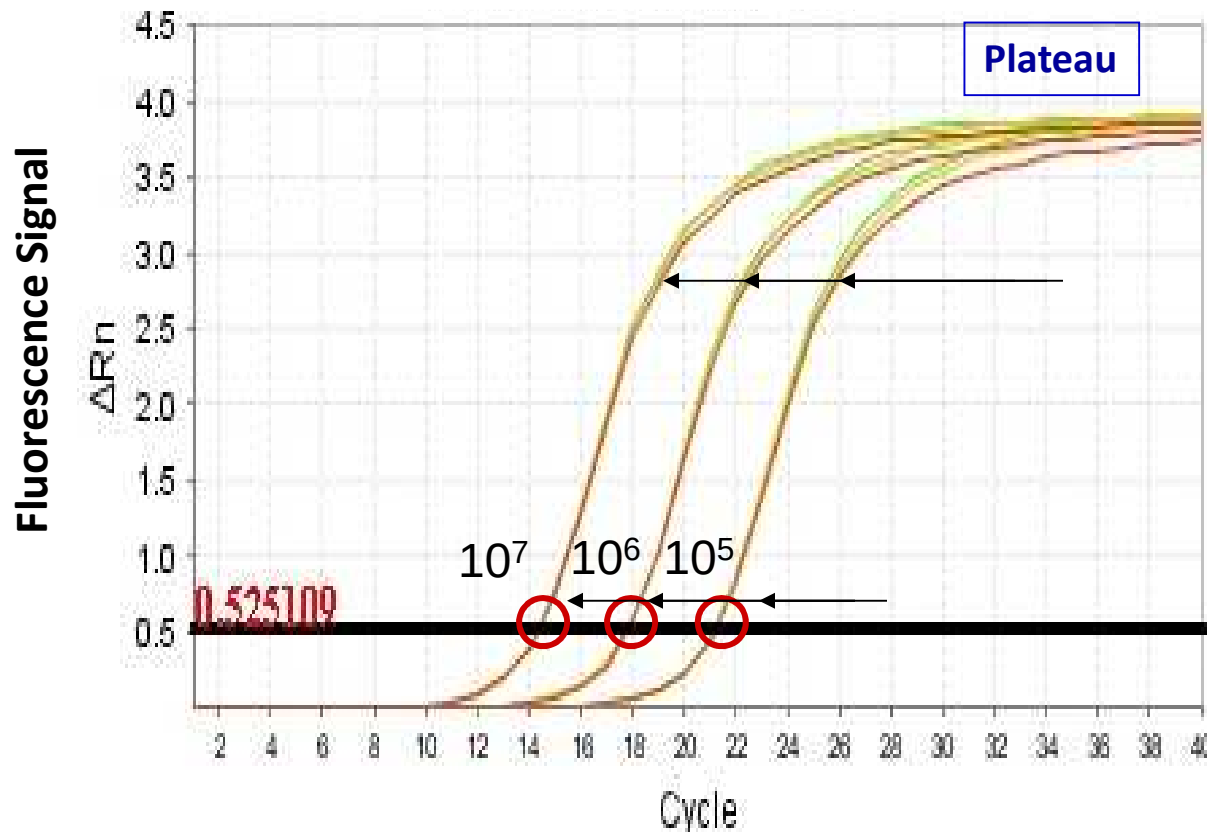
Fluorescent SYBR I



The SYBR I signal intensities correlate with DNA amplified (amplicon amount) thus the initial **sample** input amounts

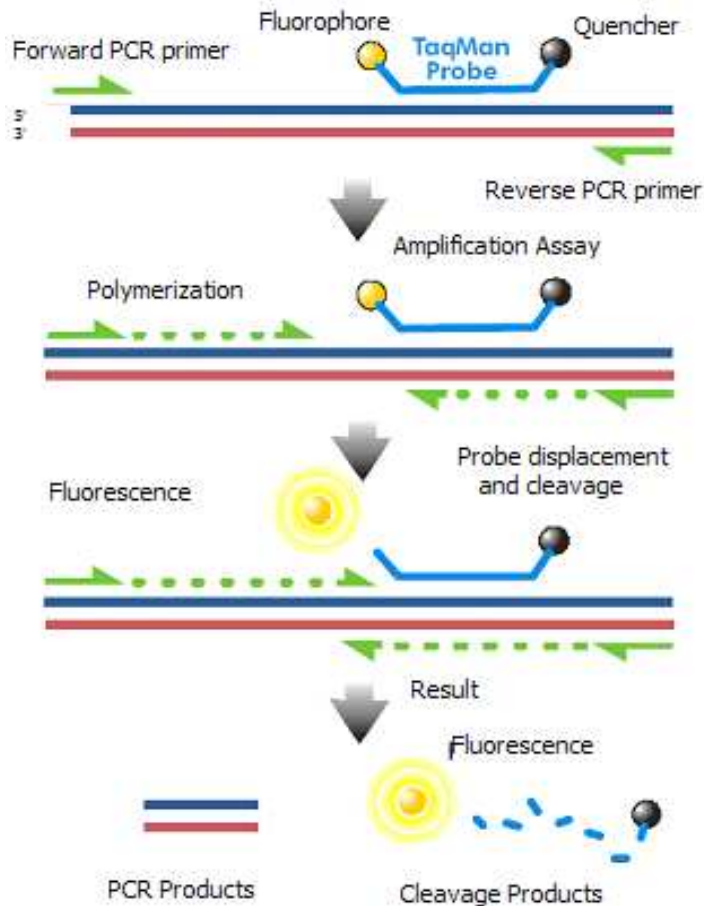
- Simple & cost saving
- **High Specificity Is Required** when using SYBR Green since SYBR I binds all double-strand DNA (non-specific or primer dimer).

Amplification Plot (Linear scale)



- Plateau phase
 - End-point PCR data collection at plateau (gel analysis)
 - Reactions varying due to reagent depletion & decreased PCR efficiencies (enzyme activity, more product competing for primer annealing)
- Exponential Phase
 - Real time PCR does early phase detection
 - proportional to input amounts
 - $2^n = \text{dilution factor}$

Hydrolysis Based Probe - - Taqman® Probe Assay



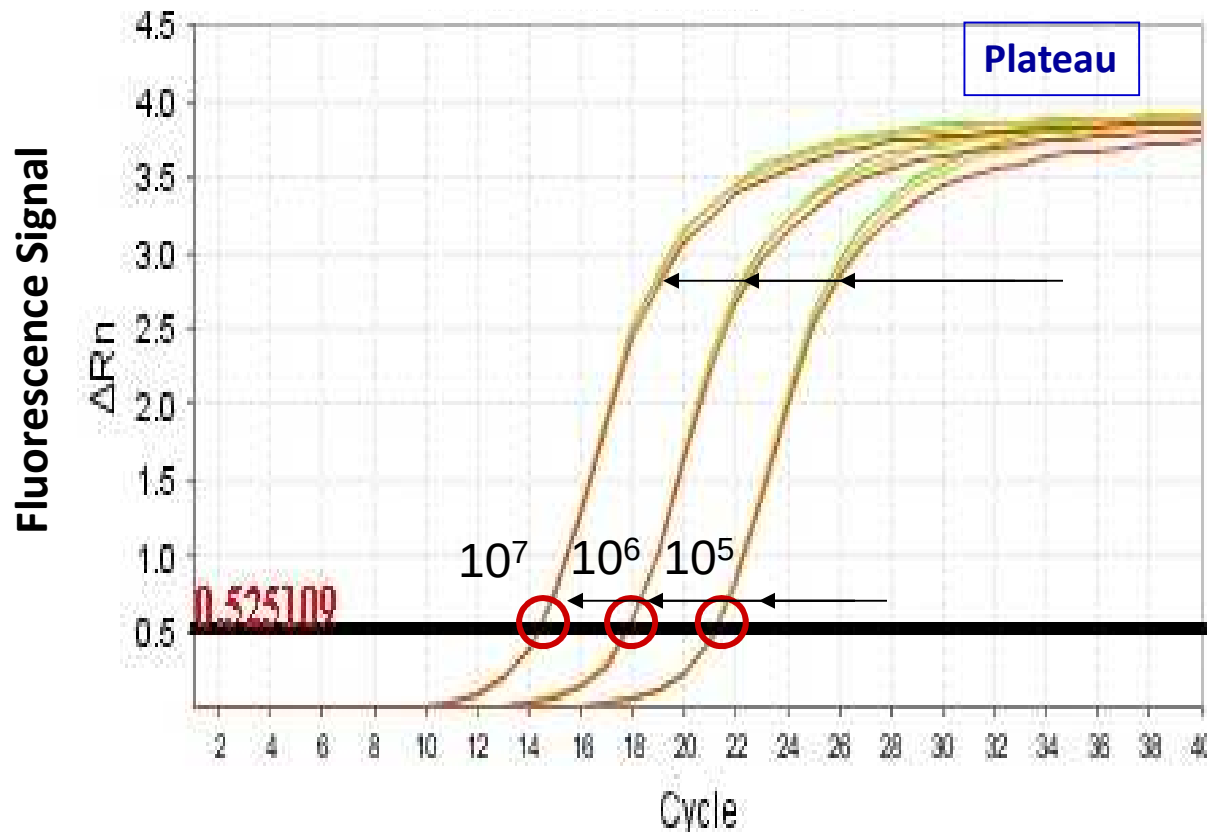
The fluorescence of the reporter dye is suppressed by the quencher

Primer binding followed by extension

**Probe cleavage by Taq to free the reporter dye thus the fluorescence intensity correlates with the initial sample input amounts.
Taq has 5' → 3' exonuclease activity**

Each amplicon needs a sequence-specific probe (cost & time)

Amplification Plot (Linear scale)



- Plateau phase
 - End-point PCR data collection at plateau (gel analysis)
 - Reactions varying due to reagent depletion & decreased PCR efficiencies (enzyme activity, more product competing for primer annealing)
- Exponential Phase
 - Real time PCR does early phase detection
 - proportional to input amounts
 - $2^n = \text{dilution factor}$



Characteristics of a good qPCR Assay

What factors do you need to address to create a good PCR Assay?

Amplification efficiency: 100% during exponential phase (template product doubles with each cycle)

Sensitivity: Able to detect down to reasonable quantities of template in 1 reaction (10-50 copies)

Specificity: 1 assay, 1 target: (no off-target amplification or primer-dimers)

- Melt-curve analysis - 1 peak, 1 product
- Agarose gel

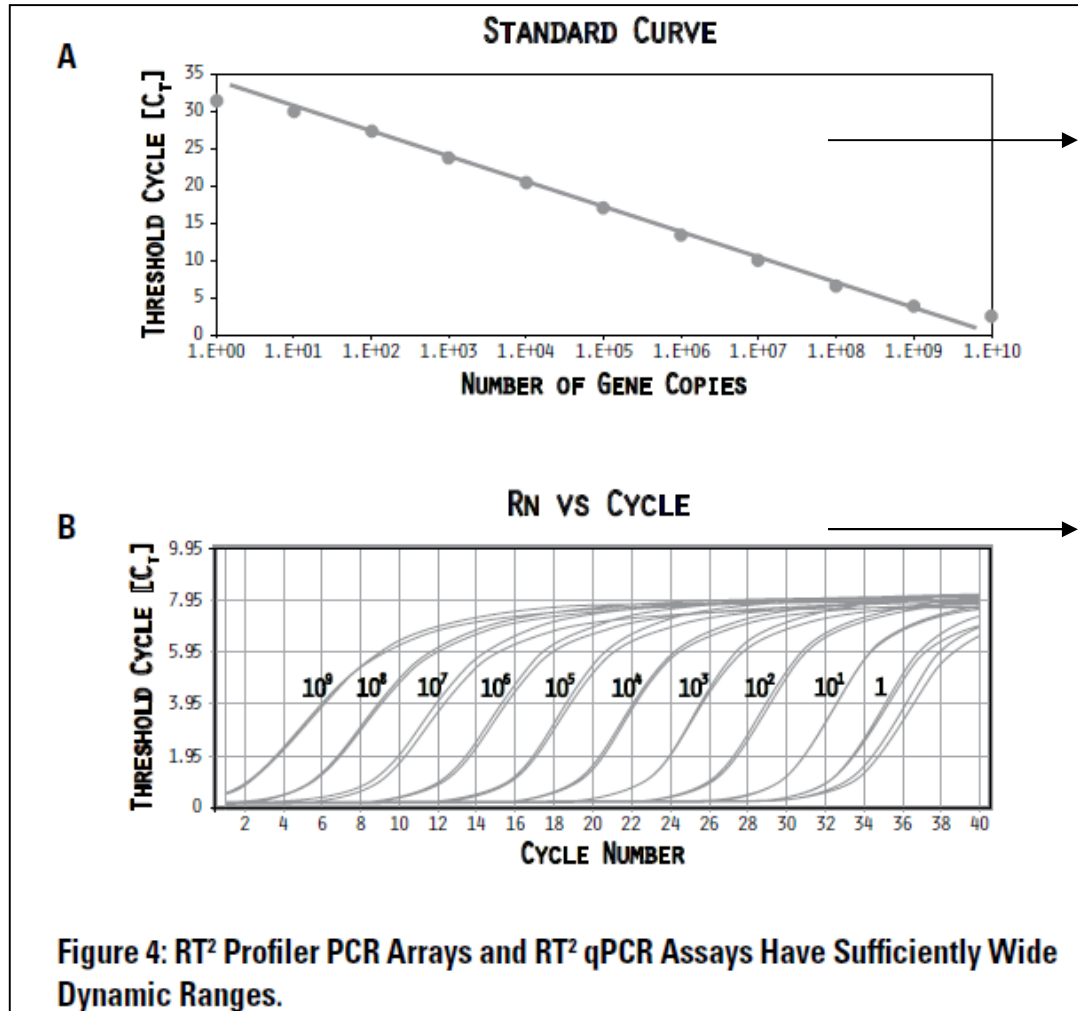
Dynamic Range: Ability to detect genes with varied expression levels, another judge of sensitivity

- 10 to 10^9 copies is ideal

Reproducibility: Confidence in your results, enables profiling of multiple genes in the same sample

- All lab members get the same results
- Technical reproducibility ensures changes seen in results are due to the biology and not the technology itself or sample handling

Characteristics of a good qPCR Assay: Amplification Efficiency

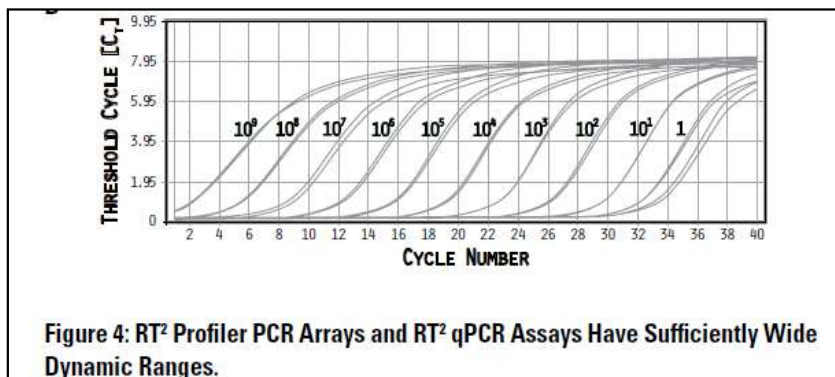
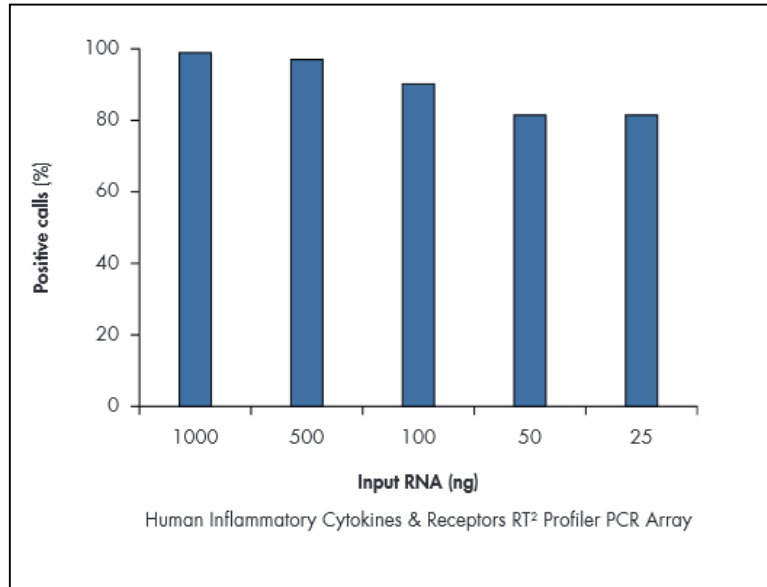


Amplification Efficiency: reliable and accurate experiment

Two Methods:

- Standard curve
 - X axis - dilution
 - Y axis - Ct value
 - Amp efficiency = $10^{(-1/\text{slope})} - 1$ * 100
- Single curve analysis
 - PCR Miner: <http://miner.ewindup.info/version2>
 - "DART": www.gene-quantification.de/DART_PCR_version_1.0.xls

Characteristics of a good qPCR Assay: Sensitivity



Sensitivity: How many copies can my assay detect?

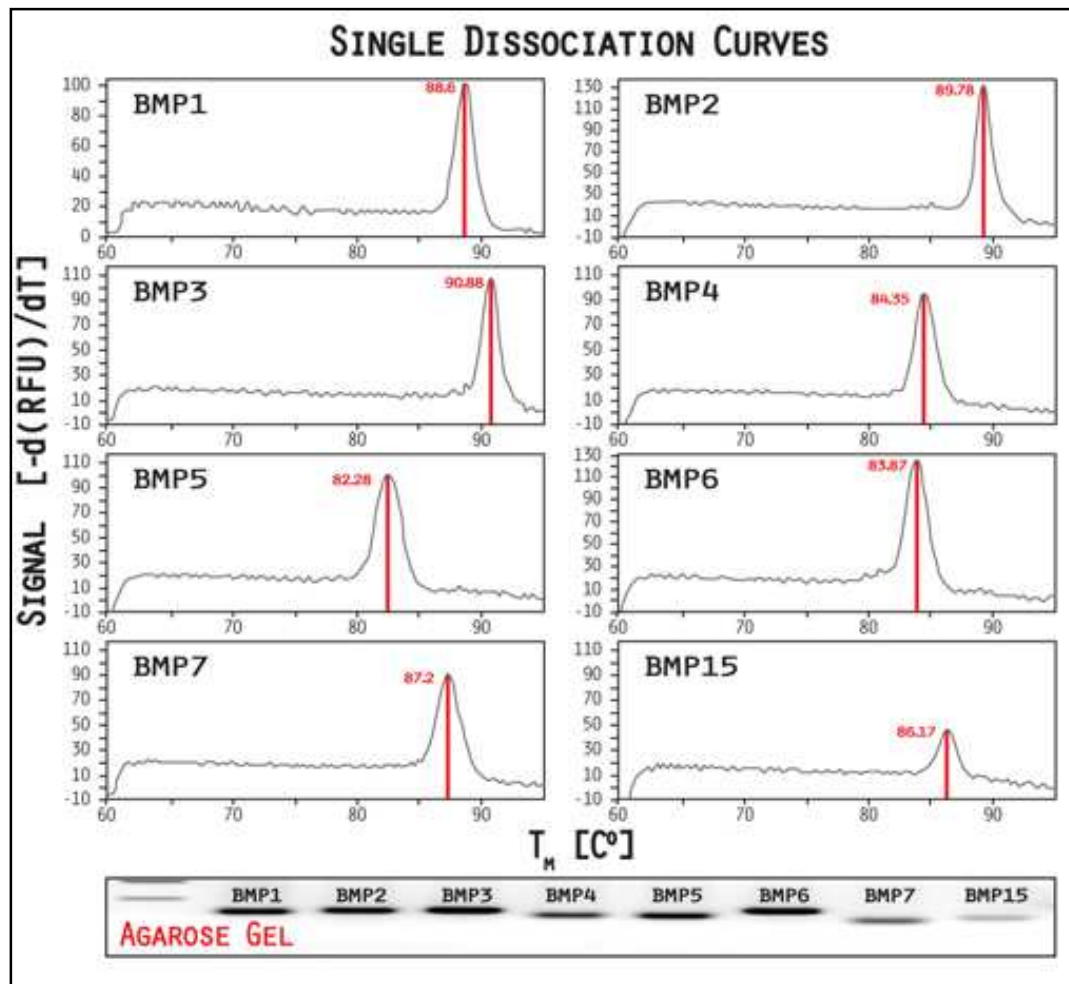
- Important for low expressed genes or where there is limited sample

Two Methods:

- Method 1: Use primers to make PCR product, T/A clone, grow-up, isolate, quantitate and use for qPCR reactions

- Method 2: Use gDNA as template and use mass of gDNA to calculate copy number and assume 1 target per genome (or actually calculate targets using bioinformatics)

Characteristics of a good qPCR Assay: Specificity

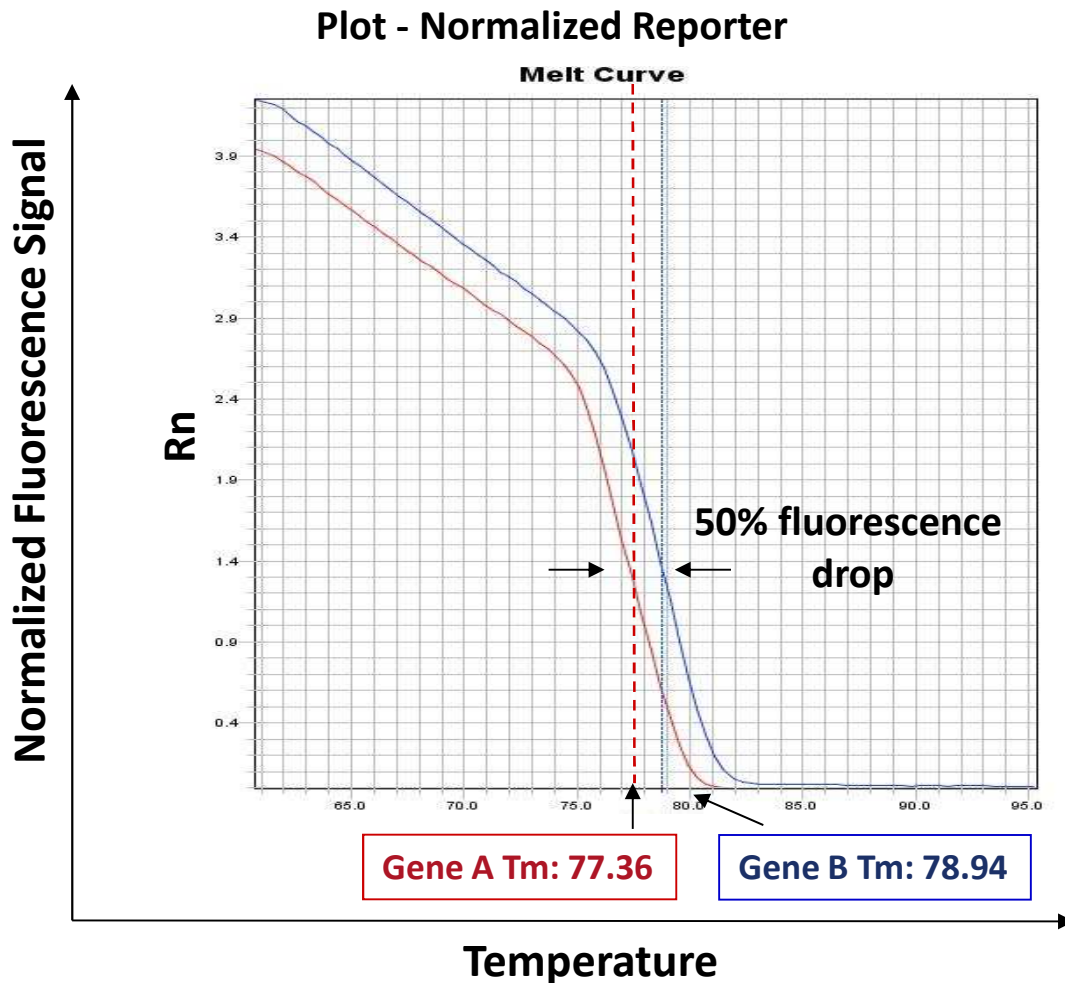


Specificity: 1 target amplified

Two Methods:

- Melt Curve analysis
 - 1 peak, 1 product
- Agarose gel
 - Band at correct size

Melting Curve Analysis

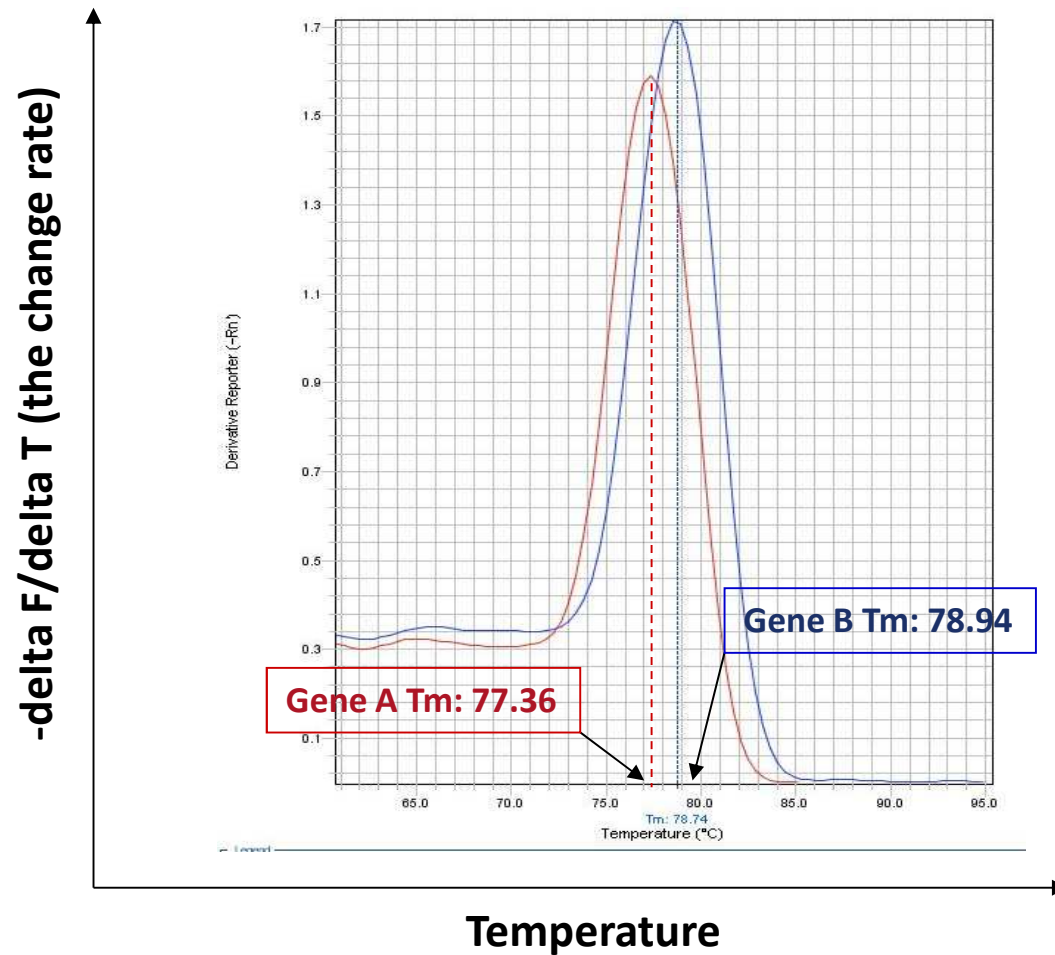


The General Program Steps

- Heat to 94°C to denature DNA
- Cooling to 60°C to let DNA double strands anneal
- Slowly heat (increase temp. to 0.2°C/sec) while plotting the fluorescent signal vs. temperature.
- As the temp increases, DNA melts, fluorescent signal should decrease.
- Significant drop in signal when 50% DNA melts.

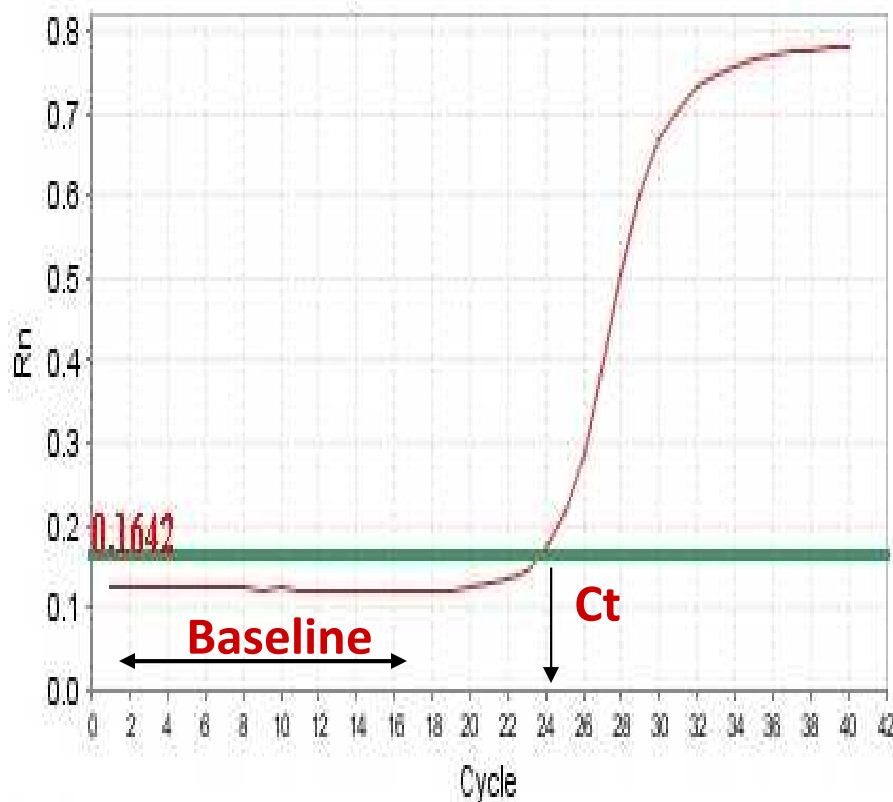
Melting Curve Analysis

Plot -1st negative Derivative Reporter



- Single melt curve of each amplicon is required for specificity validation

Linear Amplification Plot



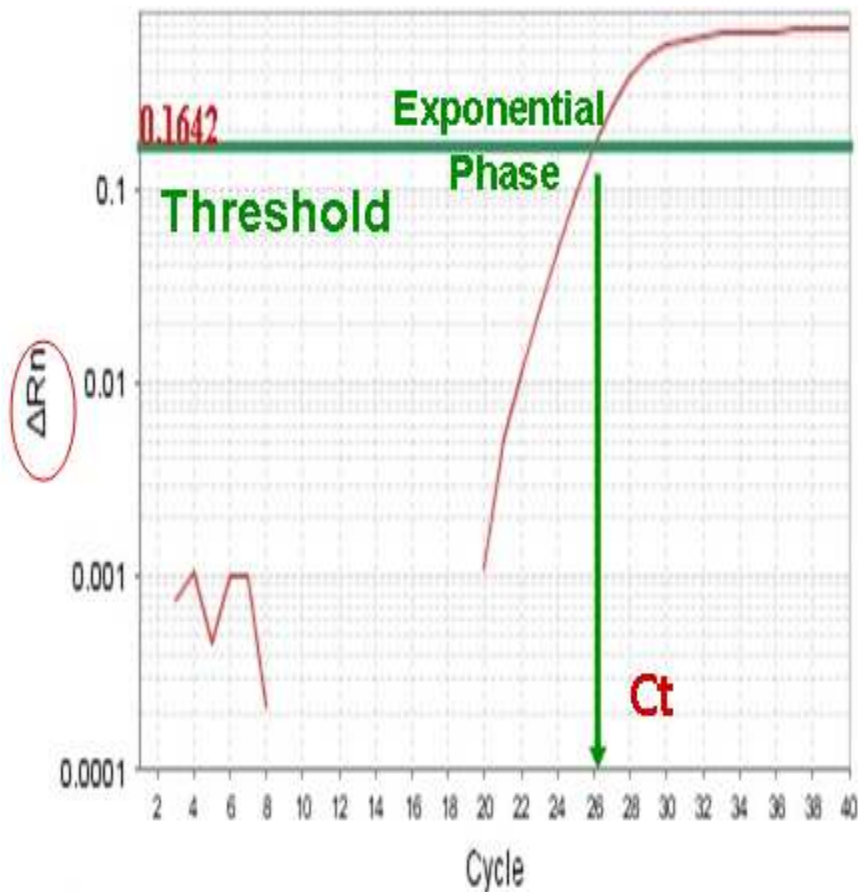
- **Automated Baseline Option**

if an instrument has a adaptive baseline function

- **Manual Baseline Option**

- (1) Use linear view of the plot
- (2) Set up the baseline reading from cycle #2 to the cycle that 2 cycles before the earliest visible amplification
- (3) Usually a baseline falls in **3-15** cycles

Log View Amplification Plot



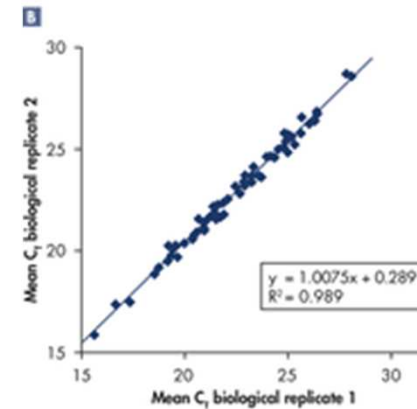
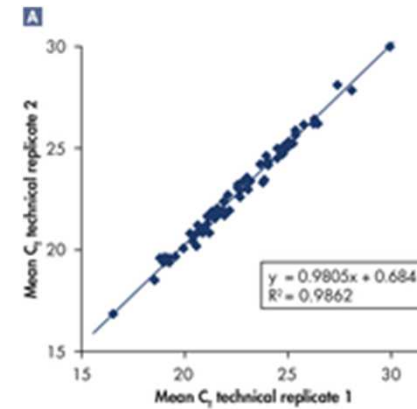
- Use **log view** of amplification plot
- Threshold should be higher than baseline (higher than the noise level)
- Threshold should be at **LOWER** 1/3 or 1/2 of the linear phase of amplification
- **Linear phase = exponential phase**
- Different runs across samples for the same experiments should have **the same threshold** for comparison

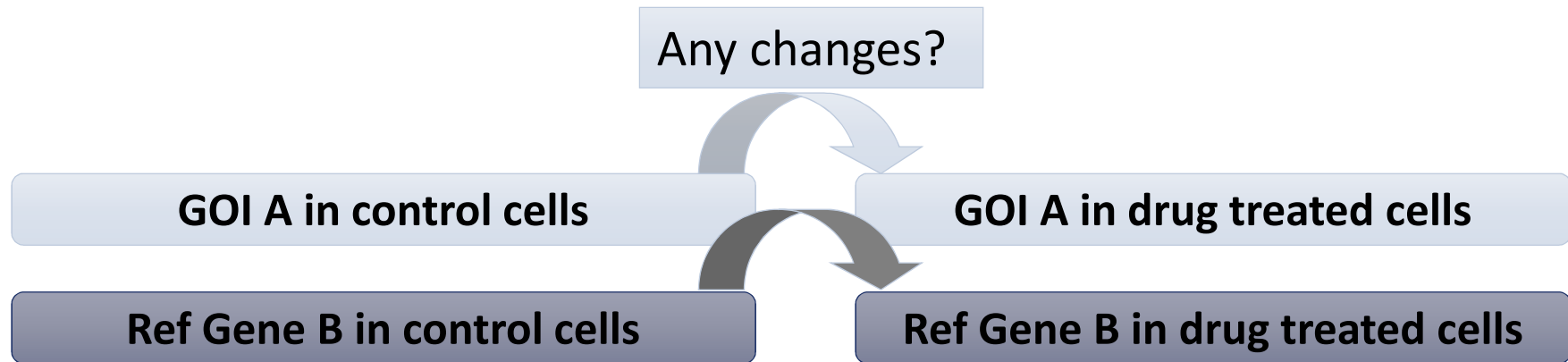
Biological Replicates: 3 different experiments

- Shows variability due to experiment

Technical replicates: 3 different measurements for same step

- Shows variability due to pipetting, machine, enzymes, etc.





Reference gene

- Expression level remains consistent under experimental conditions/different tissues
- Aimed to normalize possible variations during:
 - Sample prep & handling (e.g use the same number of cells from a start)
 - RNA isolation (RNA quality and quantity)
 - Reverse transcription efficiency across samples/experiments
 - PCR reaction set up
 - PCR reaction amplification efficiencies

Data Analysis: Commonly Used Housekeeping Genes

Table 7. Housekeeping genes commonly used as endogenous references

Gene	Gene symbol		Relative expression level*	
	Human	Mouse	Human	Mouse
18S ribosomal RNA	RRN18S	Rn18s	++++	++++
Actin, beta	ACTB	Actb	+++	+++
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Gapdh	+++	+++
Phosphoglycerate kinase 1	PGK1	Pgk1	+++	++
Peptidylprolyl isomerase A	PPIA	Ppia	+++	+++
Ribosomal protein L13a	RPL13A	Rpl13a	+++	+++
Ribosomal protein, large, P0	RPLP0		+++	
Acidic ribosomal phosphoprotein P0		Arbp		+++
Beta-2-microglobulin	B2M	B2m	++ - +++	++ - +++
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	YWHAZ	Ywhaz	++ - +++	+
Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	SDHA	Sdha	++	+
Transferrin receptor	TFRC	Tfrc	++	+
Aminolevulinate, delta-, synthase 1	ALAS1	Alas1	+	+
Glucuronidase, beta	GUSB	Gusb	+	+
Hydroxymethylbilane synthase	HMBS	Hmbs	+	++ - +++
Hypoxanthine phosphoribosyltransferase 1	HPRT1	Hprt1	+	+
TATA box binding protein	TBP	Tbp	+	+
Tubulin, beta	TUBB		+	
Tubulin, beta 4		Tubb4		+

* "+" indicates relative abundance of the transcripts.

1. Average Ct values for all gene replicates
2. Calculate ΔCt value between GOI and HKG for each experiment
3. Average ΔCt values between experiments (replicates)
4. Calculate $\Delta\Delta\text{Ct}$ values (ΔCt experiment- ΔCt control)
5. Calculate Fold Change $2^{(-\Delta\Delta\text{Ct})}$

Normalized Gene Expression Level

Any changes?

Target Gene A in control cells

Target Gene A in drug treated cells

Reference Gene B in control cells

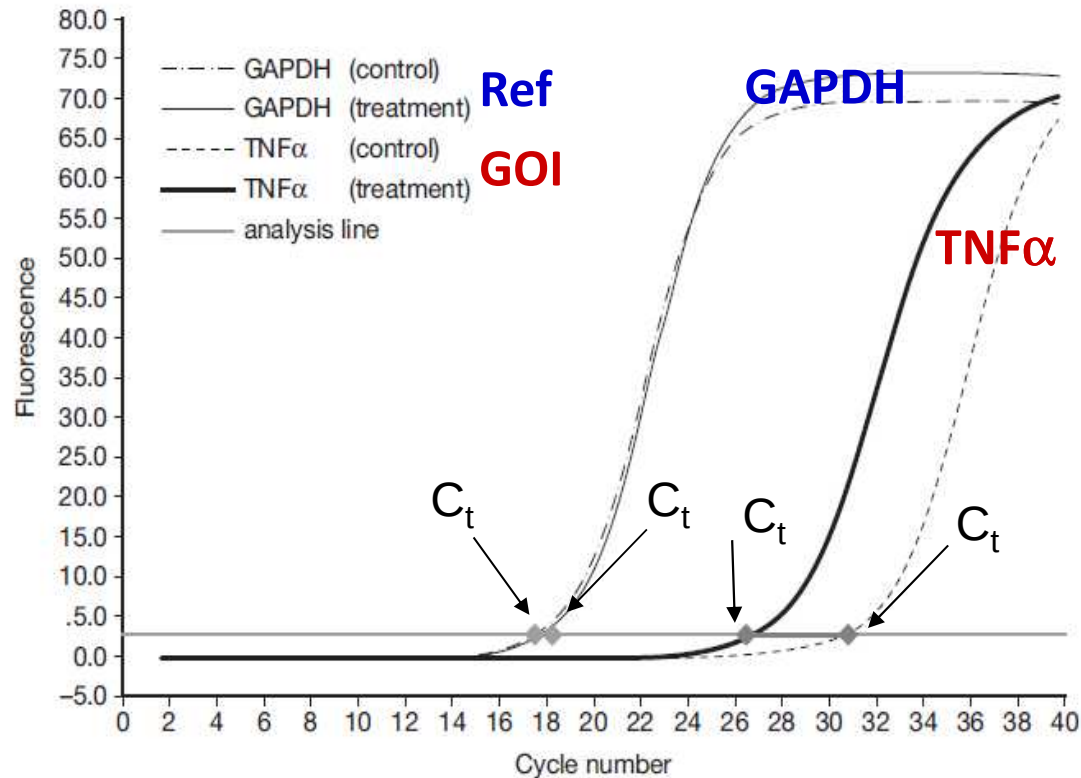
Ref Gene B in drug treated cells

➔ $\Delta Ct = Ct (\text{Target A -treated}) - Ct (\text{Ref B-treated})$

➔ $\Delta Ct = Ct (\text{Target A-control}) - Ct (\text{Ref B-control})$

➔ $\Delta\Delta Ct = \Delta Ct (\text{treated}) - Ct (\text{control})$

Normalized target gene expression level = $2^{(-\Delta\Delta Ct)}$



$$\Delta\Delta C_t = \Delta C_t (\text{TNF}\alpha_{\text{treat}} - \text{GAPDH}_{\text{treat}}) - \Delta C_t (\text{TNF}\alpha_{\text{control}} - \text{GAPDH}_{\text{control}})$$

$$\text{The fold change} = 2^{(-\Delta\Delta C_t)}$$

- 1.) Average Ct values for all gene replicates
- 17.1, 17.2, 17.2 ← qPCR replicates

	Control 1	Control 2	Control 3	Exp 1	Exp 2	Exp 3
GAPDH	17.2	18	19	17	18	17.5
TNFα	31	32.3	33.4	26	27.2	26.8

- 2.) Calculate Delta Ct value: GOI-HKG

TNFα-GAPDH	13.8	14.3	14.4	9	9.2	9.3
------------	------	------	------	---	-----	-----

- 3.) Average Delta Ct values between experiments (replicates)

Average	14.17			9.17
---------	-------	--	--	------

- 4.) Calculate Delta-Delta Ct values (Delta Ct experiment- Delta Ct control)

DD Ct	-5.00
-------	-------

- 5.) Calculate Fold Change $2^{(-\text{Delta Delta Ct})}$

Fold Change	32.00
-------------	-------

TNFα is up-regulated 32 fold in the treated cells versus the control


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The GeneGlobe Data Analysis Center is a web resource for scientists analyzing their real-time PCR or NGS data. The real-time PCR modules transform threshold cycle (C_T) values to calculated results for gene and miRNA expression, somatic mutation detection and copy number measurements. The NGS module supports the analysis of QIAGEN's GeneRead target enrichment panels. Register on QIAGEN.com to access this complimentary suite of tools to accelerate your data analysis and interpretation.

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You Selected:

Technology: RT² Profiler PCR Array

Catalog Number: PAHS-021Z

Plate Format: 96-well

Want to analyze data from a different product?
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File: No file selected.

* File must be a MS Excel Sheet (in .XLS format, not .XLSX).

Excel Templates for Formatting your Experimental Data:

[96-Well Cataloged PCR Array](#)

Instructions:



Does your data look like this?
If not, please download the appropriate Excel template to format your data correctly.

	A	B	C	D	E	F
1		Resting 6 h	Resting 6 h	Resting 6 h	6-h Stimulation	6-h Stimulation
2		Control Group	Control Group	Control Group	Test Group 1	Test Group 1
3	A01	29.08	29.02	29.27	29.89	29.56
4	A02	32.02	32.13	31.96	31.15	31.27
5	A03	33.83	34.22	33.09	31.57	31.24
6	A04	33.95	33.26	32.65	31.3	32.24
7	A05	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
8	A06	29	28.84	28.53	26.67	26.27
9	A07	Undetermined	37.05	Undetermined	37.28	35.35
10	A08	27.33	27.11	27.31	29.54	29.45
11	A09	25.52	25.6	25.81	21.26	21.29
12	A10	27.12	27.21	27.12	16.77	16.86
13	A11	35.53	36.21	37.66	33.49	36.02
14	A12	23.03	23.28	23.16	21	20.94
15	B01	34.1	34.36	32.92	35.17	35.06
16	B02	33.13	36.08	34.1	33.1	33.11
17	B03	25.3	25.36	25.3	25.02	25



Topics Covered Today

1. What is qPCR? Applications and workflow
2. qPCR for gene expression: What is the change in gene expression during differentiation?
3. Factors Critical For A Successful qPCR Assay
4. RNA purity and integrity
5. Reverse Transcription
6. qPCR in Action
7. Reporter chemistries
8. Characteristics of a good qPCR assay
9. Analyzing qPCR curves
10. Data & analysis



Upcoming webinars: July 2015

Monday	Tuesday	Wednesday	Thursday	Friday
		1	2	3
6 Molecular mechanisms of neurodegeneration* Host-pathogen interactions	7 cfDNA isolation and sample handling* NGS: introduction to technology and applications	8 miRNA biomarker discovery*	9 Practical hints for successful PCR*	10 qPCR introduction
13 Non-coding RNAs & neurodegeneration* Microbiome: from identification to characterization	14 Liquid biopsy — a new era in diagnosis* Addressing challenges of NGS workflows	15 miRNA overview*	16 Novel in-process monitoring qPCR tools* Multiplex PCR for genotyping	17 Coding & non-coding RNA analysis using qPCR
20 Circulating biomarkers for Alzheimer's disease* Innate immune system	21 QIAsymphony* Targeted NGS for cancer research	22 miRNA expression analysis and quantitation*	23 Critical factors for multiplex qPCR* Critical factors for multiplex end-point PCR	24 PCR array data analysis tutorial
27 Basics of sample prep in RNA research* Toll-like receptors in inflammation	28 Optimized NGS workflow mutation detection in cfDNA* Biomedical Genomics Workbench and IVA	29 Functional analysis of miRNA*	30 Streamline gene expression workflow* Streamlined genotyping workflow with QIAxcel	31 Service for biomarker research

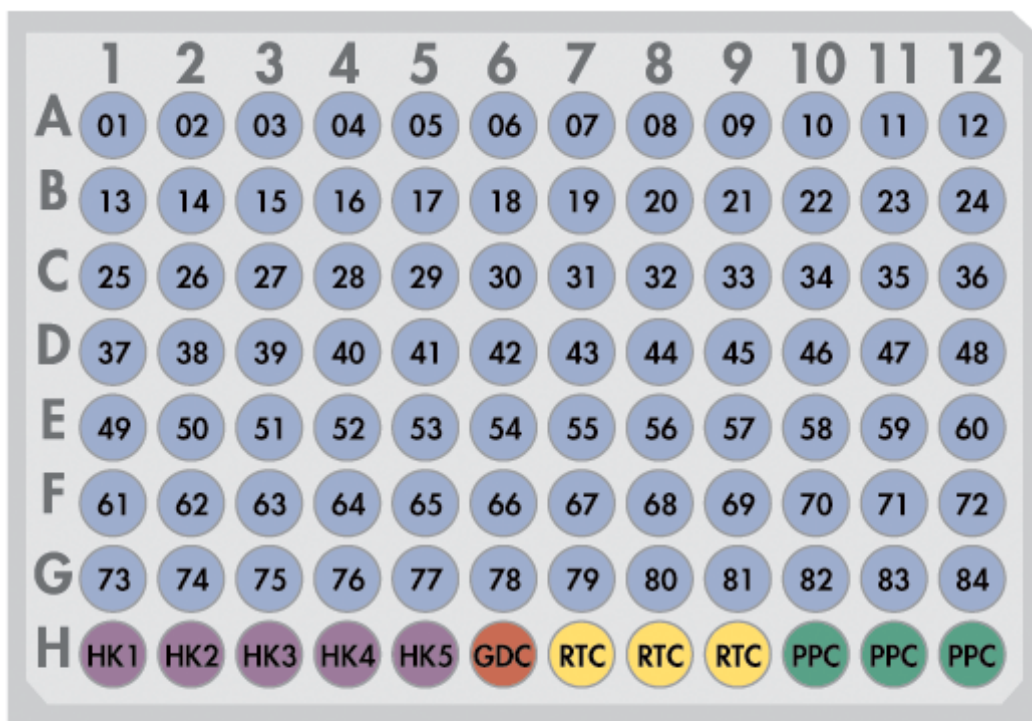
- 9:30 am EST (1:30pm GMT)*
- 1:00 pm EST (5:00pm GMT)

Register:

<http://www.qiagen.com/Knowledge-and-Support/Webinars/>

Learn about RT² Profiler PCR Arrays

Catalogued RT² Profiler by pathway & disease



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Reverse
transcription
controls

Positive
PCR controls

Pre-validated qPCR assays with controls

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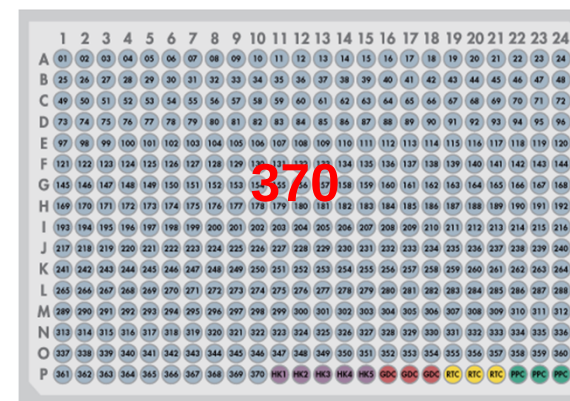


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HKG

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